

245

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
5 July 2001 (05.07.2001)

PCT

(10) International Publication Number
WO 01/48183 A2(51) International Patent Classification⁷: C12N 15/00(74) Agent: BAYLISS, Geoffrey, Cyril; Boulton Wade Tennant,
Verulam Gardens, 70 Gray's Inn Road, London WC1X
8BT (GB).

(21) International Application Number: PCT/EP00/13149

(22) International Filing Date:
22 December 2000 (22.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9930691.2 24 December 1999 (24.12.1999) GB(71) Applicant (for all designated States except US): DEV-
GEN NV [BE/BE]; Technologiepark 9, B-9052 Zwij-
naarde (BE).(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GI, GM, GR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (for US only): PLAETINCK,
Geert [BE/BE]; Pontstraat 16, B-9820 Merelbeke (BE).
MORTIER, Katherine [BE/BE]; Paddenhoek 20, B-9830
St.-Martens Latem (BE). LISSENS, Ann [BE/BE]; Tiens-
esteenweg 137, B-3010 Kessel-Lo (BE). BOGAERT,
Thierry [BE/BE]; Wolvendreef 26g, B-8500 Kortrijk
(BE).

Published:

Without international search report and to be republished
upon receipt of that report.For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

WO 01/48183 A2

(54) Title: IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

(57) Abstract: There are described ways of improving the efficiency of double stranded RNA inhibition as a method of inhibiting gene expression in nematode worms such as *C. elegans*. In particular, the invention relates to the finding that changes in the genetic background of *C. elegans* result in increased sensitivity to double-stranded RNA inhibition.

IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNAINHIBITION

5 The present invention is concerned with ways of
improving the efficiency of double stranded RNA
inhibition as a method of inhibiting gene expression
in nematode worms such as *C. elegans*. In particular,
the invention relates to the finding that the
susceptibility of nematode worms such as *C. elegans* to
10 double stranded RNA inhibition is affected by changes
in the genetic background of the worms.

It has recently been described in Nature Vol 391,
pp.806-811, February 98, that introducing double
stranded RNA into a cell results in potent and
15 specific interference with expression of endogenous
genes in the cell, which interference is substantially
more effective than providing either RNA strand
individually as proposed in antisense technology. This
specific reduction of the activity
20 of the gene was also found to occur in the nematode
worm *Caenorhabditis elegans* (*C. elegans*) when the RNA
was introduced into the genome or body cavity of the
worm.

The present inventors have utilized the double
25 stranded RNA inhibition technique and applied it
further to devise novel and inventive methods of (i)
assigning functions to genes or DNA fragments which
have been sequenced in various projects, such as, for
example, the human genome project and which have yet
30 to be accorded a particular function, and (ii)
identifying DNA responsible for conferring a
particular phenotype. Such methods are described in
the applicant's co-pending application number WO
00/01846. Processes for introducing RNA into a living
35 cell, either *in vivo* or *ex vivo*, in order to inhibit
expression of a target gene in that cell are

CONFIRMATION COPY

additionally described in WO 99/32619.

Several different experimental approaches can be used to introduce double-stranded RNA into nematode worms in order to achieve RNA interference *in vivo*.

5 One of the most straightforward approaches is simple injection of double-stranded RNA into a body cavity. A more elegant solution is to feed the nematodes on food organisms, generally bacteria, which express a double stranded RNA of the appropriate sequence,
10 corresponding to a region of the target gene.

The present inventors have now determined that the phenomenon of RNA interference in nematodes following ingestion of food organisms capable of expressing double-stranded RNA is dependent both on
15 the nature of the food organism and on the genetic background of the nematodes themselves. These findings may be exploited to provide improved methods of double-stranded RNA inhibition.

Therefore, according to a first aspect of the
20 present invention there is provided a method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism which is capable of producing a double-stranded RNA structure having a nucleotide sequence
25 substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the nematode has a non wild-type genetic background selected to provide increased sensitivity to RNA interference as compared to wild
30 type.

Caenorhabditis elegans is the preferred nematode worm for use in the method of the invention although the method could be carried out with other nematodes and in particular with other microscopic nematodes,
35 preferably microscopic nematodes belonging to the genus *Caenorhabditis*. As used herein the term "microscopic" nematode encompasses nematodes of

approximately the same size as *C. elegans*, being of the order 1mm long in the adult stage. Microscopic nematodes of this approximate size can easily be grown in the wells of a multi-well plate of the type generally used in the art to perform mid- to high-throughput screening.

It is an essential feature of this aspect of the invention that the nematode has a non wild-type genetic background which confers greater sensitivity to RNA interference phenomena (abbreviated herein to RNAi) as compared to the equivalent wild type nematodes. As illustrated in the accompanying examples, introduction of double-stranded RNA (abbreviated herein to dsRNA) into a non wild-type strain according to the invention results in greater inhibition of expression of the target gene. Depending on the nature of the target gene, this greater level of inhibition may be detectable at the phenotypic level as a more pronounced phenotype.

The nematode having non wild-type genetic background may, advantageously, be a mutant strain. Mutations which have the effect of increasing susceptibility of the nematode to RNAi may, for example, affect the stability of dsRNA or the kinetics of dsRNA turnover within cells of the worm or the rate of uptake of dsRNA synthesised by a food organism. Suitable mutant strains include mutant strains exhibiting knock-out or loss-of-function mutations in one or more genes encoding proteins involved in RNA synthesis, RNA degradation or the regulation of these processes.

In one preferred embodiment, the nematode is a mutant strain, more preferably a mutant *C. elegans*, which exhibits reduced activity of one or more nucleases compared to wild-type. Suitable strains include mutant strains exhibiting knock-out or loss-of-function mutations in one or more genes encoding

nucleases, such as RNases. A particularly preferred example is the *nuc-1* strain. This mutant *C. elegans* strain is known per se in the art.

5 In a second preferred embodiment, the nematode is a mutant strain, more preferably a mutant *C. elegans*, which exhibits increased gut uptake compared to wild-type. Particularly preferred examples of such strains are the so-called *C. elegans* gun mutants described herein. In a still further embodiment, the nematode
10 may be a transgenic worm comprising one or more transgenes which increase gut uptake relative to wild-type.

The term "increased gut uptake" as used herein is taken to mean increased uptake of foreign particles
15 from the gut lumen and may encompass both increased gut permeability and increased gut molecular transport compared to wild-type *C. elegans*.

C. elegans feeds by taking in liquid containing its food (e.g. bacteria). It then spits out the
20 liquid, crushes the food particles and internalises them into the gut lumen. This process is performed by the muscles of the pharynx. The process of taking up liquid and subsequently spitting it out is called pharyngeal pumping. Once the food particles have been
25 internalised via pharyngeal pumping their contents must cross the gut itself in order to reach target sites in the worm. There are multiple factors which effect the uptake of compounds from the gut lumen to the surrounding tissues. These include the action of
30 multi-drug resistance proteins, multi-drug resistance related proteins and the P450 cytochromes as well as other enzymes and mechanisms available for transport of molecules through the gut wall.

C. elegans mutants which exhibit increased uptake
35 of foreign molecules through the gut may be obtained from the *C. elegans* mutant collection at the C.

C. elegans Genetic Center, University of Minnesota, St Paul, Minnesota, or may be generated by standard methods. Such methods are described by Anderson in Methods in Cell Biology, Vol 48, "C. elegans: Modern biological analysis of an organism" Pages 31 to 58. Several selection rounds of the PCR technique can be performed to select a mutant worm with a deletion in a desired gene. Alternatively, a population of worms could be subjected to random mutagenesis and worms exhibiting the desired characteristic of increased gut uptake selected using a phenotypic screen, such as the dye uptake method described herein.

As an alternative to mutation, transgenic worms may be generated with the appropriate characteristics. Methods of preparing transgenic worms are well known in the art and are particularly described by Craig Mello and Andrew Fire, Methods in Cell Biology, Vol 48, Ed. H.F. Epstein and D.C. Shakes, Academic Press, pages 452-480.

Worms exhibiting the desired characteristics of increased gut uptake can be identified using a test devised by the inventors based on uptake of a marker precursor molecule which is cleaved by the action of enzymes present in the gut lumen to generate a marker molecule which produces a detectable signal, such as fluorescence. A suitable marker precursor molecule is the fluorescent dye precursor BCECF-AM available from Molecular Probes (Europe BV), Netherlands. This dye only becomes fluorescent when cleaved by esterases and maintained at a pH above 6. The pH of the gut lumen is usually 5 or below. Thus, any BCECF-AM taken up through the pharynx into the gut lumen is not fluorescent until cleaved and the cleaved portion has entered the cells surrounding the lumen which are at a higher pH. Thus, this dye is able to quickly identify mutant or otherwise modified worms which have increased gut transport or permeability. There is a

gradual increase in fluorescence in the tissues surrounding the gut while the gut lumen remains dark. The fluorescence can be detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Specific examples of gun mutant strains isolated using this procedure which may be used in the method of the invention are strains bg77, bg84, bg85 and bg86, although it is to be understood that the invention is in no way limited to the use of these specific strains. The *C. elegans* gun mutant strain bg85 was deposited on 23 December 1999 at the BCCM/LMG culture collection, Laboratorium Voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000, Gent, Belgium under accession number LMBP 5334CB. The phrase "the bg85 mutation" as used herein refers to the specific mutation(s) present in the bg85 strain which is/are responsible for conferring the gun phenotype.

It is also within the scope of the invention to use a non wild-type nematode strain, preferable a *C. elegans* strain, having multiple mutations which affect sensitivity to RNAi. A preferred type of multiple mutant is one having at least one mutation which results in reduced nuclease activity compared to wild type and at least one mutation which results in increased gut uptake compared to wild type. An example of such a mutant is a *C. elegans* strain having the *nuc-1* mutation and at least one further gun mutation. As exemplified herein, double mutants having the *nuc-1* mutation and a gun mutation exhibit enhanced sensitivity to RNAi as compared to either *nuc-1* or gun single mutants.

For the avoidance of doubt, where particular characteristics or properties of nematode worms are described herein by relative terms such as "enhanced"

or "increased" or "decreased" this should be taken to mean enhanced, increased or decreased relative to wild-type nematodes. In the case of *C. elegans*, wild-type is defined as the N2 Bristol strain which is well known to workers in the *C. elegans* field and has been extremely well characterised (see Methods in Cell Biology, Volume 48, *Caenorhabditis elegans: Modern biological analysis of an organism*, ed. by Henry F. Epstein and Diane C. Shakes, 1995 Academic Press; The nematode *Caenorhabditis elegans*, ed. by William Wood and the community of *C. elegans* researchers., 1988, Cold Spring Harbor Laboratory Press; *C. elegans* II, ed. by Donald L. Riddle, Thomas Blumenthal, Barbara J. Meyer and James R. Priess, 1997, Cold Spring Harbor Laboratory Press). The N2 strain can be obtained from the *C. elegans* Genetic Center, University of Minnesota, St Paul, Minnesota, USA.

The food organism for use in the above aspect of the invention is preferably a bacterium such as, for example, a strain of *E.coli*. It will, however, be appreciated that any other type of food organism on which nematodes feed and which is capable of producing dsRNA could be used. The food organism may be genetically modified to express a double-stranded RNA of the appropriate sequence, as will be understood with reference to the examples included herein. One convenient way in which this may be achieved in a bacterial food organism is by transforming the bacterium with a vector comprising a promoter or promoters positioned to drive transcription of a DNA sequence to RNA capable of forming a double-stranded structure. Examples of such vectors will be further described below.

The actual step of feeding the food organism to the nematode may be carried out according to procedures known in the art, see WO 00/01846.

Typically the feeding of the food organisms to the nematodes is performed on standard agar plates commonly used for culturing *C. elegans* in the laboratory. However, the step of feeding the food
5 organism to the nematodes may also be carried out in liquid culture, for example in the wells of 96-well microtitre assay plates.

The inventors have further observed that variations in the food organism can result in enhanced
10 *in vivo* RNAi when the food organism is ingested by a nematode worm.

Accordingly, in a further aspect the invention provides a method of inhibiting expression of a target gene in a nematode worm comprising feeding to said
15 nematode worm a food organism capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the food organism carries a
20 modification selected to provide increased expression or persistence of the doubled-stranded RNA compared to a food organism which does not carry the modification.

The modification present in the food organism can be any modification which results in increased
25 expression of the dsRNA or in increased persistence of the dsRNA. Suitable modifications might include mutations within the bacterial chromosome which affect RNA stability and/or degradation or mutations which have a direct effect on the rate of transcription. In
30 a preferred embodiment, the food organism is an RNase III minus *E. coli* strain, or any other RNase negative strain.

According to a still further aspect of the invention there is provided a method of inhibiting
35 expression of a target gene in a nematode worm comprising introduction of a DNA capable of producing a double-stranded RNA structure having a nucleotide

sequence substantially identical to a portion of said target gene in said nematode, wherein the nematode is one which exhibits increased gut uptake compared to wild type.

5 In addition to exhibiting increased sensitivity to RNAi following feeding with food organisms capable of expressing a dsRNA, nematodes which exhibit increase gut uptake as described herein also show increased uptake of DNA molecules capable of producing
10 double-stranded RNA structures following ingestion into a nematode.

In a preferred embodiment, the DNA is in the form of a vector comprising a promoter or promoters orientated to relative to a sequence of DNA such that
15 they are capable of driving transcription of the said DNA to make RNA capable of forming a double-stranded structure upon binding of an appropriate RNA polymerase to the promoter or promoters.

Several different arrangements of promoters may
20 be used in such a vector. In a first arrangement a DNA fragment corresponding to a region of the target gene is flanked by two opposable polymerase-specific promoters which are preferably identical. Transcription from the opposable promoters produces
25 two complementary RNA strands which can anneal to form an RNA duplex. The plasmid pGN1 described herein is an example of a vector comprising two opposable T7 promoters flanking a multiple cloning site for insertion of a DNA fragment of the appropriate
30 sequence, corresponding to a region of a target gene. pGN8 is an example of a vector derived from pGN1 containing a fragment of the *C. elegans unc-22* gene. In an alternative arrangement, DNA fragments corresponding to a region of the target gene may be
35 placed both in the sense and the antisense orientation downstream of a single promoter. In this case, the sense/antisense fragments are co-transcribed to

generate a single RNA strand which is self-complementary and can therefore form an RNA duplex.

In both of the above arrangements, the polymerase-specific T3, T7 and SP6 promoters, all of which are well known in the art, are useful for driving transcription of the RNA. Expression from these promoters is dependent on expression of the cognate polymerase. Advantageously, the nematode itself may be adapted to express the appropriate polymerase. Expression of the polymerase may be general and constitutive, but could also be regulated under a tissue-specific promoter, an inducible promoter, a temporally regulated promoter or a promoter having a combination of such characteristics. Transgenic *C. elegans* strains harboring a transgene encoding the desired polymerase under the control of an appropriately-regulated promoter can be constructed according to methods known *per se* in the art and described, for example, by Craig Mello and Andrew Fire in *Methods in Cell Biology*, Vol 48, Ed. H. F. Epstein and D. C. Shakes, Academic Press, pp 452-480.

The advantage of adapting the nematode to express the required polymerase is that it is possible to control inhibition of expression of the target gene in a tissue-specific and/or temporally specific manner by placing expression of the polymerase under the control of an appropriately regulated promoter.

Introduction of DNA into nematodes in accordance with the method of the invention can be achieved using a variety of techniques, for example by direct injection into a body cavity or by soaking the worms in a solution containing the DNA. If the DNA is in the form of a vector as described herein, e.g. a plasmid harboring a cloned DNA fragment between two flanking T7 promoters, then dsRNA corresponding to this DNA fragment will be formed in the nematode resulting in down regulation of the corresponding gene. The

introduced DNA can form an extrachromosomal array, which array might result in a more catalytic knock-out or reduction of function phenotype. The DNA might also become integrated into the genome of the nematode, resulting in the same catalytic knock out or reduction of function phenotype, but which is stably transmittable.

In each aspect of the invention, the double-stranded RNA structure may be formed by two separate complementary RNA strands or a single self-complementary strand, as described above. Inhibition of target gene expression is sequence-specific in that only nucleotide sequences corresponding to the duplex region of the dsRNA structure are targeted for inhibition.

It is preferred to use dsRNA comprising a nucleotide sequence identical to a portion of the target gene, although RNA sequences with minor variations such as insertions, deletions and single base substitutions may also be used and are effective for inhibition. It will be readily apparent that 100% sequence identity between the dsRNA and a portion of the target gene is not absolutely required for inhibition and the phrase "substantially identical" as used herein is to be interpreted accordingly. Generally sequences which are substantially identical will share at least 90%, preferably at least 95% and more preferably at least 98% nucleic acid sequence identity. Sequence identity may be conveniently calculated based on an optimal alignment, for example using the BLAST program accessible at WWW.ncbi.nlm.nih.gov.

The invention will be further understood with reference to the following non-limiting Examples, together with the accompanying Figures in which:

Figure 1 is a plasmid map of the vector pGN1

containing opposable T7 promoters flanking a multiple cloning site and an ampicillin resistance marker.

5 Figure 2 is a plasmid map of the vector pGN8 (a genomic fragment of the *C. elegans unc-22* gene cloned in pGN1).

10 Figure 3 is a plasmid map of the vector pGN29 containing two T7 promoters and two T7 terminators flanking *Bst*XI sites. This vector permits cloning of DNA fragments linked to *Bst*XI adaptors.

15 Figure 4 is a plasmid map of the vector pGN39 containing two T7 promoters and two T7 terminators flanking attR recombination sites (based on the Gateway™ cloning system of Life Technologies, Inc).

20 Figure 5 is a plasmid map of the vector pGX22 (a fragment of the *C. elegans* gene C04H5.6 cloned in pGN29).

25 Figure 6 is a plasmid map of the vector pGX52 (a fragment of the *C. elegans* gene K11D9.2b cloned in pGN29).

Figure 7 is a plasmid map of the vector pGX104 (a fragment of the *C. elegans* gene Y57G11C.15 cloned in pGN29).

30 Figure 8 is a plasmid map of the vector pGZ8 (a fragment of the *C. elegans* gene T25G3.2 cloned in pGN39).

35 Figure 9 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the

plasmid pGX22.

Figure 10 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the plasmid pGX52.

Figure 11 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the plasmid pGXG28.

Figure 12 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the plasmid pGX104

Example 1

Influence of genetic background on the efficiency of RNAi in *C. elegans*.

5 Introduction

Various different *C. elegans* strains were fed with different bacteria, to test the possibility of RNAi by feeding *C. elegans* with bacteria that produce dsRNA. The possibility of DNA delivery and dsRNA delivery has
10 previously been envisaged by using different bacterial strains. In this experiment the importance of the *C. elegans* strain as receptor of the dsRNA is also shown.

For this experiment the following *E. coli* strains were
15 used:

1. MC1061: F-*araD139* Δ (*ara-leu*) 7696 *galE15 galK16*
 Δ (*lac*)X74 *rps1* (*Str^r*) *hsdR2* (*r_k⁻ m_k⁺*) *mcrA mcrB1*
- regular host for various plasmids,
20 - Wertman et al., (1986) Gene 49:253-262,
- Raleigh et al., (1989) in Current Protocols in Molecular Biology eds. Ausubel et al, Publishing associates and Wiley Interscience; New York. Unit 1.4.
- 25 2. B21(DE3): F- *ompT(lon)* *hsdS_B* (*r_B⁻ m_B⁻*; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene.
- regular host for IPTG inducible T7 polymerase expression,
30 - Studier et al. (1990) Meth. Enzymol. 185:60-89
3. HT115 (DE3): F- *mcrA mcrB* IN(*rrnD-rrnE*) 1 λ -*rnc14::tr10* (DE3 lysogen: *lacUV5*
35 promoter-T7polymerase)
- host for IPTG inducible T7 polymerase

expression,

- RNaseIII-,

- Fire A, Carnegie Institution, Baltimore, MD,
Pers. Comm.

5

For this experiment the following *C. elegans* strains
were used:

1. *C. elegans* N2: regular WT laboratory strain
- 10 2. *C. elegans* nuc-1(el393): *C. elegans* strain with a
reduced endonuclease activity (>95%); condensed
chromatin persists after programmed cell death;
ingested (bacterial) DNA in the intestinal lumen
15 is not degraded. Several alleles are described:
el392 (strong allele: has been used for the
experiments described below); n887 (resembles
el392) and n334 (weaker allele)
- Stanfield et al. (1998) East Coast Worm
20 meeting abstract 171,
- Anonymous, Worm Breeder's Gazette 1(1):17b
Hevelone et al. (1988) Biochem. Genet. 26:447-461
- Ellis et al., Worm breeder's Gazette 7(2):44
- Babu, Worm Breeder's gazette 1(2):10
25 - Driscoll, (1996) Brain Pathol. 6:411-425
- Ellis et al., (1991) Genetics 129:79-94

For this experiment the following plasmids were used:

- 30 pGN1: A vector encoding for ampicillin resistance,
harbouring a multiple cloning site between two
convergent T7 promoters.
- pGN8: pGN1 containing a genomic fragment of *unc-22*.
35 Decreased *unc-22* expression via RNAi results in a
"twitching" phenotype in *C. elegans*.

Experimental conditions

12-well micro-titer plates were filled with approximately 2 ml of NGM agar per well (1 litre of NGM agar: 15g Agar, 1g peptone, 3g NaCl, 1ml cholesterol solution (5 mg/ml in EtOH), with sterile addition after autoclaving of 9.5 ml 0.1M CaCl₂, 9.5 ml 0.1 ml MgSO₄, 25 ml 1M KH₂PO₄/K₂HPO₄ buffer pH 6 and 5 ml nystatin solution (dissolved 10 mg/ml in 1:1 EtOH:CH₃COONH₄ 7.5 M).

10

The dried plates were spotted with approximately 50 µl of an overnight culture of bacteria. When IPTG induction was required, 50 µl of a 10 mM stock solution of IPTG was dropped on top of the bacteria lawn, and incubated at 37°C for approximately 4 hours. Individual nematodes at the L4 growth stage were then placed in single wells. In each well 4 nematodes, and the plates were further incubated at 20°C for 6 days to allow offspring to be formed. The F1 offspring of the seeded nematodes were tested for the twitching phenotype.

15

20

Results

Table 1: Percentage of the offspring that show the twitching phenotype

5	MC1061	N2	<i>nuc-1</i>
	pGN1	0%	0%
	pGN1 + IPTG	0%	0%
	pGN8	0%	0%
	pGN8 + IPTG	0%	0%
10	BL21 (DE3)		
	pGN1	0%	0%
	pGN1 + IPTG	0%	0%
	pGN8	20% (+)	>90% (++)
	pGN8 + IPTG	20% (+)	>90% (++±)
15	HT115 (DE3)		
	pGN1	0%	0%
	pGN1 + IPTG	0%	0%
	pGN8	50% (±)	>90% (++)
20	pGN8 + IPTG	80% (++)	>90% (+++)

%: percentage twitchers

+: weak twitching

++: twitching

+++: strong twitching

Conclusions

The experiment with *E. coli* MC1061 shows that no twitching could be observed in this experiment.

30 Neither the N2 nematodes nor the *nuc-1* nematodes showed any twitchers. This is to be expected as *E. coli* MC1061 does not produce any T7 RNA polymerase, and hence the *unc-22* fragment cloned in pGN8 is not

expressed as dsRNA.

5 The experiment with *E. coli* strain BL21(DE3) and
nematode strain N2 shows expected results. BL21(DE3)
harbouring plasmid pGN1 does not result in any
twitching as the pGN1 vector is an empty vector. BL21
(DE3) harbouring PGN8 results in the expression of
unc-22 dsRNA. When this dsRNA is fed to the N2
nematode (indirectly by feeding with the bacteria that
10 produce the dsRNA), this results in a twitching
phenotype, indicating that the dsRNA is able to pass
the gut barrier and is able to perform its interfering
activity.

15 Surprisingly the RNAi effect of the unc-22 dsRNA was
even more pronounced in *C. elegans* strain *nuc-1* than
in the wild type N2 strain. Although one may expect
that the *nuc-1* mutation results in the non-degradation
or at least in a slower degradation of DNA, as the
20 NUC-1 protein is known to be involved in DNase
activity, we clearly observe an enhancement of the
RNAi induced phenotype in *C. elegans* with a *nuc-1*
background. The *nuc-1* mutation has not been cloned
yet, but it has been described that the gene is
25 involved in nuclease activity, and more particularly
DNase activity. If the NUC-1 protein is a nuclease, it
may also have activity on nuclease activity on dsRNA,
which would explain the enhanced RNAi phenotype. The
nuc-1 gene product may be a nuclease, or a regulator
30 of nuclease activity. As the mode of action of RNAi is
still not understood, it is also possible that the
NUC-1 protein is interfering in the mode of action of
RNAi. This would explain why a *nuc-1* mutant is more
sensitive to RNAi.

35

The experiment with the *E. coli* strain HT115 (DE3)

confirms the experiments with the BL21(DE3) strain. The RNA interference observed with the unc-22 dsRNA is even higher. In comparison with strain BL21(DE3) this could be expected, as HT115(DE3) is a RNase III minus strain, and hence is expected to produce larger amounts of dsRNA, resulting in more prominent RNAi. This indicates further that the RNAi observed in this experiment is the result of the dsRNA produced by the bacteria fed to the *C. elegans*. Feeding *C. elegans* nuc-1 with HT115(DE3) harbouring pGN8 also results in higher RNA interference phenotype than feeding the same bacteria to *C. elegans* wild-type strain N2. Once again this indicates that improved RNAi can be realised using a nuclease negative *C. elegans* and more particularly with a with the *C. elegans* nuc-1 (e1392) strain.

Summary

RNA interference can be achieved in *C. elegans* by feeding the worms with bacteria that produce dsRNA. The efficiency of this RNA interference is dependent both on the *E. coli* strain and on the genetic background of the *C. elegans* strain. The higher the level of dsRNA production in the *E. coli*, the more RNAi is observed. This can be realised by using efficient RNA expression systems such as T7 RNA polymerase and RNase negative strains, such as RNaseIII minus strains. In this example the level of dsRNA production varied: HT115(DE3)>BL21(DE3)>MC1061.

RNA interference is high in *C. elegans* strains that are nuclease negative, or that are influenced in their nuclease activity. This can be realised by using a mutant strain such as *C. elegans* nuc-1. In this example the sensitivity to RNAi varied: *C. elegans* nuc-1 >> *C. elegans* N2

Example 2

Improved RNAi by feeding dsRNA producing bacteria in selected *C. elegans* strains-Comparison of the *nuc-1* strain with several mutants which show improved gut uptake. (designated herein 'gun' mutants). Strains bg77, bg78, bg83, bg84, bg85, bg86, bg87, bg88 and bg89 are typical gun mutant *C. elegans* strains isolated using selection for increased gut uptake (gun phenotype) with the marker dye BCECF-AM.

Experimental conditions:

- 12-well micro-titer plates were filled with approximately 2ml of NGM agar (containing 1ml/l of ampicillin (100µg/ml) and 5 ml of 100mM stock IPTG) per well
- the dried plates were spotted with 25µl of an overnight culture of bacteria (BL21DE3/HT115DE3) containing the plasmids pGN1 (T7prom-T7prom) or pGN8 (T7prom-unc-22-T7prom)
- individual nematodes at the L4 growth stage were then placed in single wells, one nematode per well
- the plates were incubated at 20°C for 6 days to allow offspring to be formed
- the adult F1 offspring of the seeded nematodes were tested for the twitching phenotype

Results:

Table 2:

	20°C/6d	pGN1 HT115DE3	pGN8 BL2DE3	pGN8 HT115DE3	
5	N2	0	1	1	
	nuc-1	0	1-2	3	
	bg77	0	1-2	3	
	bg78	0	1	1-2	
	bg83	0	1	1	
10	bg84	0	1-2	3	
	bg85	0	1	2-3	
	bg86	0	1	2	
	bg87	0	1	1	
	bg88	0	1	1	
15	bg89	0	1	1	

figure legend:

0 = no twitching

20 1 = no to weak phenotype

2 = clear phenotype

3 = strong phenotype

25 **Conclusions**

- bacterial strain HT115(DE3) shows a better RNAi sensitivity than bacterial strain BL21(DE3)
- the *nuc-1* *C. elegans* strain is a better strain than the Wild-type N2 strain for RNAi sensitivity
- 30 - various gun mutants (improved gut uptake mutants) and more particularly the gun mutant strains bg77, bg84, bg85, bg86 show improved sensitivity to RNAi compared to Wild-type.

A double mutant *C. elegans* strain (*nuc-1/gun*) shows even greater sensitivity to RNAi compared to wild-type:

5 Double mutants were constructed to test the prediction that *gun/nuc* mutants would even show more enhanced RNAi sensitivity. As an example, the crossing strategy with *gun* strain bg85 is shown, similar crosses can be conducted with other *gun* strains, such
10 as bg77, bg84 and bg86.

P0 cross: *gun*(bg85) x WT males

15 F1 cross: *nuc-1* x *gun*(bg85)/+ males

F2 cross: *nuc-1* x *gun*(bg85)/+; *nuc-1/0* males (50%)
nuc-1 x +/+; *nuc-1/0* males (50%)

20 F3 single: *gun*(bg85)/+; *nuc-1* hermaphrodites (25%)
+/+; *nuc-1* hermaphrodites (75%)

F4 single: *gun*(bg85); *nuc-1* (1/4 of every 4th plate high staining with BCECF)

25 F5 retest: *gun*(bg85); *nuc-1* (100% progeny of F4 singled high staining with BCECF)

To select for the *gun* phenotype, the fluorescence precursor BCECF-AM is used (obtainable from Molecular probes). The precursor BCECF-AM is cleaved by
30 esterases present in the gut of the worm to generate the dye BCECF which is fluorescent at pH values above 6. This allows selection for worms that have a *gun* phenotype. BCECF-AM is taken up through the pharynx
35 into the gut lumen and is not fluorescent until it has been cleaved, and the BCECF portion has entered the

cells surrounding the lumen. Wild-type worms will show slower or no increase in BCECF fluorescence.

5 **Example 3**

Improved RNAi feeding in liquid culture using *nuc-1*(el393) *C. elegans*.

Introduction

10 N2 and *nuc-1* *C.elegans* strains were fed with bacteria producing dsRNAs that give lethal phenotypes via RNAi. For this example RNAi was performed in liquid culture instead of on agar plates. We show here for a number of genes that the RNAi effect is more penetrant using
15 the *nuc-1* strain than the N2 strain, and that RNAi can be performed in liquid.

For this experiment the following *E.coli* strains were used:

20

1. HT115 (DE3): F- *mcrA mcrb* IN(*rrnD-rrnE*) 1 λ -
 rncl4::trl0 (DE3 lysogen: lacUV5 promoter -T7
 polymerase)
 - host for IPTG inducible T7 polymerase expression
25 - RNaseIII⁻
 - Fire A, Carnegie Institution, Baltimore, MD,
 Pers. Comm.

25

For this experiment, following *C. elegans* strains were
30 used:

30

1. *C. elegans* N2: regular WT laboratory strain
2. *C. elegans nuc-1*(el393): *C. elegans* strain with a
35 reduced endonuclease activity (>95%); condensed
 chromatin persists after programmed cell death;

35

ingested (bacterial) DNA in the intestinal lumen is not degraded. Several alleles are described:

- 5 e1392 (strong allele: has been used for the experiments described below); n887 (resembles e1392) and n334 (weaker allele)
- Stanfield et al. (1998) East Coast Worm meeting abstract 171
 - Anonymous, Worm Breeder's Gazette 1(1):17b
 - Hevelone et al. (1988) Biochem. Genet. 26:447-461
 - 10 - Ellis et al., Worm breeder's Gazette 7(2):44
 - Babu, Worm Breeder's gazette 1(2):10
 - Driscoll, (1996) Brain Pathol. 6:411-425
 - Ellis et al., (1991) Genetics 129:79-94

15

For this experiment, the following plasmids that all give lethal phenotypes in *C. elegans* via RNAi were used:

- 20 pGX22: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid C04H5.6 corresponding to a member of the RNA helicase family.

- 25 pGX52: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid K11D9.2b corresponding to sarco/endoplasmic Ca²⁺ ATPase also known as SERCA.

- 30 pGZ18: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid T25G3.2 corresponding to a chitin like synthase gene.

- 35 pGX104: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid Y57G11C.15 corresponding to sec-61, a transport protein.

Experimental conditions

- 5 - 1 ml overnight cultures of HT115 (DE3) bacteria containing the plasmids pGX22, pGX52, pGZ18 or pGX104 respectively were pelleted and resuspended in S-complete medium, containing 1ml/l of ampicillin (100 µg/ml) and 1ml/l of 1000mM IPTG.
- 10 - 10 µl of this bacterial solution was transferred to a 96-well microtiter plate already filled with 100 µl S-complete containing 1ml/l of ampicillin (100 µg/ml) and 1ml/l of 1000mM IPTG.
- 15 - 3 nematodes at the L1 growth stage of N2 and nuc-1 strain were then placed in single wells, 3 L1's per well. Per experimental set up, 16 wells were used (n=16).
- 20 - the plates were incubated at 25°C for 5 days to allow offspring to be formed.
- the plates were visually checked and the following phenotypes could be scored per individual well:
- 25 **no effect:** L1's developed to adults and gave normal offspring.
- no F1 offspring:** L1's developed to adults and gave no offspring.
- 30 **acute lethal:** original L1 did not mature and died.

Results

- 35 The results of this experiment are illustrated graphically in Figures 9 to 12. Data are expressed as

a percentage of the total (n=16) on the y-axis for both N2 and *nuc-1* strains.

Conclusions

5 The following genes were tested in this liquid RNAi assay:

- C04H5.6: an RNA helicase. RNAi of this gene interferes with the generation of offspring.
- 10 - SERCA: a sarco/endoplasmic Ca^{2+} ATPase. A strong RNAi phenotype causes an acute lethal phenotype. A less penetrant RNAi effect results in loss of offspring.
- T25G3.2: a chitin like synthase gene. RNAi of
15 this gene causes dead eggs.
- sec-61: a transport protein. A strong RNAi phenotype causes an acute lethal phenotype. A less penetrant RNAi effect results in loss of offspring.
- 20 - RNAi can be performed under liquid conditions.

As in the previous examples this set of experiments shows that the *nuc-1* *C. elegans* strain is more sensitive to RNAi than the wild-type N2 strain. This
25 is most clear for less penetrant phenotypes such as SERCA and chitin synthase. For strong RNAi phenotypes like the helicase and Sec-61 the difference between the N2 wild-type strain and the *nuc-1* stain is less pronounced.

30

Example 4**Cloning of pGX22, pGX52, pGZ18 and pGX104 for RNAi**

A set of primers for each gene was designed on the basis of sequence data available in the publicly accessible *C. elegans* sequence database (Acedb).

The cosmid names relate to:

1. **C04H5.6**=member of RNA helicase
2. **K11D9.2b**=SERCA
3. **Y57G11C.15**=transport protein sec-61
4. **T25G3.2**=chitin synthase like

The following primer sequences were designed:

1. **C04H5.6F** 5'-TGCTCAGAGAGTTTCTCAACGAACC-3'
C04H5.6R 5'-CAATGTTAGTTGCTAGGACCACCTG-3'
2. **K11D9.2bF** 5'-CAGCCGATCTCCGTCTTGTG-3'
K11D9.2bR 5'-CCGAGGGCAAGACAACGAAG-3'
3. **Y57G11C.15F** 5'-ACCGTGGTACTCTTATGGAGCTCG-3'
Y57G11C.15R 5'-TGCAGTGGATTGGGTCTTCG-3'
4. **T25G3.2F**
5'-GGGGACAAGTTTGTACAAAAAGCAGGCTATGCCAAGTACATGTCGATTGCG-3'
- T25G3.2R**
5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGAGAAGCATTCGAGAGTTTG-3'

PCR was performed on genomic DNA of N2 strain *C. elegans* to give PCR products of the following sizes:

- 1326bp for C04H5.6
- 1213bp for K11D9.2b

1024bp for Y57G11C.15

1115bp for T25G3.2

- 5 The PCR fragments of C04H5.6, K11D9.2b and Y57G11C.15 were linked to *Bst*XI adaptors (Invitrogen) and then cloned into the pGN29 vector cut with *Bst*XI. pGN29 contains two T7 promoters and two T7 terminators flanking a cloning site which is adapted for facilitated cloning of PCR fragments, comprising a
- 10 stuffer DNA flanked by two *Bst*XI sites (see schematic Figure 3). The resulting plasmids were designated pGX22 (C04H5.6), pGX52 (K11D9.2b) and pGX104 (Y57G11C.15).
- 15 The PCR fragment of T25G3.2 was cloned into pGN39 via recombination sites based on the GATEWAY™ cloning system (Life Technologies, Inc). pGN39 contains two T7 promoters and two T7 terminators flanking a cloning site which facilitates "High Throughput" cloning based
- 20 on homologous recombination rather than restriction enzyme digestion and ligation. As shown schematically in Figure 4, the cloning site comprises *att*R1 and *att*R2 recombination sites from bacteriophage lambda flanking a gene which is lethal to *E. coli*, in this
- 25 case the *ccdB* gene. This cloning site is derived from the Gateway™ cloning system commercially available from Life Technologies, Inc. The Gateway™ cloning system has been extensively described by Hartley et al. in WO 96/40724 (PCT/US96/10082).

Example 5

Selecting *C. elegans* mutations for increased gut uptake (gun) using marker dye BCECF-AM and *unc-31* as background.

5

The screen was performed in *unc-31*(e928) mutant background, to ensure high amounts of dye in the gut lumen, since *unc-31* mutations show constitutive pharyngeal pumping. The dye (BCECF-AM: 2',7' bis (2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethylester), obtained from Molecular Probes, is cleaved by intracellular esterases. Fluorescence accumulates in the gut cells upon passage through the apical gut membrane.

15

Mutagenesis

Day 1: *unc-31* L4 staged worms were mutagenised with EMS (final concentration 50 mM) for 4 hours

Day 2: P0 was divided over several large agar plates

Day 6: F1's were collected and dropped on large plates. The number of eggs the F1's laid were checked every hour and the F1's were removed when 10-20 eggs per F1 were counted

Day 10: F2 adults were collected and screened with BCECF-AM. Mutations with increased staining of the gut cells after 15-30 minutes exposure to the dye were selected and singled on small agar plates.

30

About 50 initial positives gave progeny which was retested with BCECF-AM (2x) and leucine CMB (1x) 9 of the 50 strains were kept (2 strains : 3 times positive, 7 other strains : twice positive)

35

Table 3: Isolation of mutations for increased staining with BCECF-AM

Total P0	Total F1	Total F2	screened chromosomes	number of strains isolated
(counted)	(estimated)	(calculated)	(estimated)	(counted)
2251	55618	222472	100000	9

Outcrossing, backcrossing and double construction

- 10 1. backcrossing *unc-31; gun* --> *unc-31; gun*
 - *unc-31; gun* x WT males
 - singled 2x5 WT hermaphrodites F1s (*unc-31/+;gun/+*)
 - singled 50 WT hermaphrodites F2s (1/4 homozygous)
 - select strains segregating 1/4 *unc*
- 15 - stain *unc* strains with BCECF-AM
 - from positive strains pick *unc* homozygous
 - retest 100 % *unc* strains with BCECF-AM
 - kept 1 strain (backcrossed)
- 20 2. *unc-31* background was crossed out-->+; *gun*
 - *unc-31; gun* x WT males
 - singled 2x5 WT hermaphrodites F1s (*unc-31/+;gun/+*)
 - singled 50 WT hermaphrodites F2s (1/4 homozygous)
 - select strains which did not segregate *unc* F3s
- 25 anymore
 - stain non *unc* strains with BCECF-AM
 - 7 positive strains were retested with BCECF-AM and finally 1 was selected and kept (outcrossed)
- 30 3. +; *gun* (1x outcrossed) were 2 times backcrossed-->+; *gun* (3x backcrossed)
 - *gun* x WT males
 - WT hermaphrodites x F1 males (*gun/+*)
 - singled 10 WT hermaphrodites F2s (1/2 heterozygous)
- 35 - singled 50 WT hermaphrodites F3s (1/8 homozygous)

- 31 -

- stain strains with BCECF-AM- retested positives with BCECF-AM and finally 1 was selected and kept

4. *gun* (3x backcrossed) were crossed with *nuc-1(X)*
 5 mutant--> *gun; nuc-1*
 - *gun* x WT males
 - *nuc-1* x *gun/+* males
 - *nuc-1* x *gun/+; nuc-1/0* or *+/+; nuc-1/0* males
 - singled 10 WT hermaphrodite progeny (*nuc-1*
 10 homozygous, $\frac{1}{2}$ heterozygous *gun*)
 - singled 40 WT hermaphrodite progeny (1/8 homozygous *gun*)
 - stain strains with BCECF-AM
 - retested positives with BCECF-AM and finally 1 was
 15 selected and kept

Table 6: Strains derived from *gun* mutations

	<i>gun</i>	<i>unc-31; gun</i>		<i>unc-31; gun</i>		+; <i>gun</i>		<i>gun; nuc-1</i>
		original isolate		backcrossed (1x)		outcrossed (1x)		from 3x b.c.
	allele number	isolation number	strain number	isolation number	strain number	isolation number	strain number	strain number
20	bg77	31.4	UG 510	31.4.46.1	UG 556	31.4.34	UG 563	UG 674
25	bg78	37.5	UG 511	37.5.46.4	UG 557	37.5.15	UG 564	UG 675
	bg83	10.2	UG 543	10.2.11	UG 600	10.2.21	UG 586	UG 676
	bg84	7.2	UG 544	7.2.10	UG 601	7.2.15	UG 589	UG 677
	bg85	11.5	UG 545	11.5.29.2	UG 602	2x b.c.	UG 717	UG 775
	bg86	42.1	UG 546	42.1.4.5	UG 603	42.1.18	UG 587	UG 678
30	bg87	7.1	UG 547	7.1.8.3	UG 604	7.1.22	UG 585	UG 679
	bg88	5.3	UG 548	5.3.9	UG 605	5.3.18	UG 584	UG 680
	bg89	23.4	UG 549	23.4.13.5	UG 606	23.4.3	UG 588	UG 671

35

SEQUENCE LISTING:

SEQ ID NO: 1 complete sequence of pGN1

5 SEQ ID NO: 2 complete sequence of pGN8

SEQ ID NO: 3 complete sequence of pGN29

SEQ ID NO: 4 complete sequence of pGN39

10 SEQ ID NO: 5 complete sequence of pGX22

SEQ ID NO: 6 complete sequence of pGX52

15 SEQ ID NO: 7 complete sequence of pGX104

SEQ ID NO: 8 complete sequence of pGZ8

SEQ ID NO: 9 primer C04H5.6F

20 SEQ ID NO: 10 primer C04H5.6R

SEQ ID NO: 11 primer K11D9.2bF

25 SEQ ID NO: 12 primer K11D9.2bR

SEQ ID NO: 13 primer Y57G11C.15F

SEQ ID NO: 14 primer Y57G11C.15R

30 SEQ ID NO: 15 primer T25G3.2F

SEQ ID NO: 16 primer T25G3.2R

Claims:

1. A method of inhibiting expression of a target gene in a nematode worm comprising feeding to
5 said nematode worm a food organism which is capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the nematode
10 has a non wild-type genetic background selected to provide increased sensitivity to RNA interference as compared to wild type.
2. A method as claimed in claim 1 wherein the
15 nematode is a microscopic nematode.
3. A method as claimed in claim 2 wherein the nematode is from the genus *Caenorhabditis*.
- 20 4. A method as claimed in claim 3 wherein the nematode is *C. elegans*.
5. A method as claimed in any one of claims 1 to 4 wherein the nematode has a mutant genetic
25 background.
6. A method as claimed in claim 5 wherein the nematode is a mutant strain which exhibits reduced activity of one or more nucleases compared to wild
30 type.
7. A method as claimed in claim 6 wherein the nematode is *C. elegans* strain *nuc-1*.
- 35 8. A method as claimed in claim 5 wherein the nematode is a mutant strain which exhibits increased

gut uptake compared to wild type.

9. A method as claimed in claim 8 wherein the nematode is mutant *C. elegans* strain bg85.

5

10. A method as claimed in claim 5 wherein the nematode is a mutant strain having at least one mutation which results in reduced nuclease activity compared to wild type and at least one mutation which results in increased gut uptake compared to wild type.

10

11. A method as claimed in claim 10 wherein the nematode is a mutant *C. elegans* strain having the *nuc-1* mutation and the bg85 mutation.

15

12. A method as claimed in any one of the preceding claims wherein the food organism has been engineered to express a double-stranded RNA.

20

13. A method as claimed in any one of the preceding claims wherein the food organism is a bacterium.

25

14. A method as claimed in claim 13 wherein the food organism is *E. coli*.

30

15. A method as claimed in any one of the preceding claims wherein the food organism has been genetically modified to express a double-stranded RNA having a nucleotide sequence substantially identical to a portion of said target gene.

35

16. A method as claimed in claim 15 wherein the food organism contains a DNA vector, the vector comprising a promoter or promoters orientated relative to a DNA sequence such that they are capable of

initiating transcription of said DNA sequence to RNA capable of forming a double-stranded structure upon binding of an appropriate RNA polymerase to said promoter or promoters.

5

17. A method as claimed in claim 25 wherein the vector comprises two promoters flanking the DNA sequence.

10

18. A method as claimed in claim 26 wherein the two promoters are identical.

15

19. A method as claimed in claim 25 wherein the vector comprises a single promoter and further comprises said DNA sequence in a sense and an antisense orientation relative to said promoter.

20

20. A method as claimed in any one of claims 16 to 20 wherein the nematode or the food organism is adapted to express an RNA polymerase capable of initiating transcription from said promoter or promoters.

25

21. A method as claimed in any one of claims 16 to 20 wherein the RNA polymerase is T7, T3 or SP6 polymerase.

30

22. A method as claimed in any one of claims 1 to 21 wherein the step of feeding said food organism to said nematode worm is carried out in liquid culture.

35

23. A method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a

portion of said target gene following ingestion of the food organism by the nematode, wherein the food organism carries a modification selected to provide increased expression or persistence of the doubled-stranded RNA compared to a food organism which does not carry the modification.

24. A method as claimed in claim 23 wherein the food organism is a bacterium.

10

25. A method as claimed in claim 24 wherein the bacterium is an *E. coli* strain.

26. A method as claimed in claim 25 wherein the *E. coli* strain is an RNase III minus strain or any other RNase negative strain.

15

27. A method as claimed in any one of claims 23 to 26 wherein the step of feeding said food organism to said nematode worm is carried out in liquid culture.

20

28. A method of inhibiting expression of a target gene in a nematode worm comprising introduction of a DNA capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene in said nematode, wherein the nematode is one which exhibits increased gut uptake compared to wild type.

25

30

29. A method as claimed in claim 28 wherein the nematode is a microscopic nematode.

30. A method as claimed in claim 29 wherein the nematode is from the genus *Caenorhabditis*.

35

31. A method as claimed in claim 30 wherein the

nematode is *C. elegans*.

32. A method as claimed in any one of claims 28
to 31 wherein the nematode has a mutant genetic
5 background.

33. A method as claimed in claim 32 wherein the
nematode is mutant *C. elegans* strain bg85.

10 34. A method as claimed in any one of claims 28
to 33 wherein the DNA capable of producing a double-
stranded RNA structure is a vector comprising a
promoter or promoters orientated relative to a DNA
sequence such that they are capable of initiating
15 transcription of said DNA sequence to RNA capable of
forming a double-stranded structure upon binding of an
appropriate RNA polymerase to said promoter or
promoters.

20 35. A method as claimed in claim 34 wherein the
vector comprises two promoters flanking the DNA
sequence.

25 36. A method as claimed in claim 35 wherein the
two promoters are identical.

30 37. A method as claimed in claim 34 wherein the
vector comprises a single promoter and further
comprises said DNA sequence in a sense and an
antisense orientation relative to said promoter.

35 38. A method as claimed in any one of claims 34
to 37 wherein the nematode is adapted to express an
RNA polymerase capable of initiating transcription
from said promoter or promoters.

39. A method as claimed in any one of claims 34

to 38 wherein the RNA polymerase is T7, T3 or SP6 polymerase.

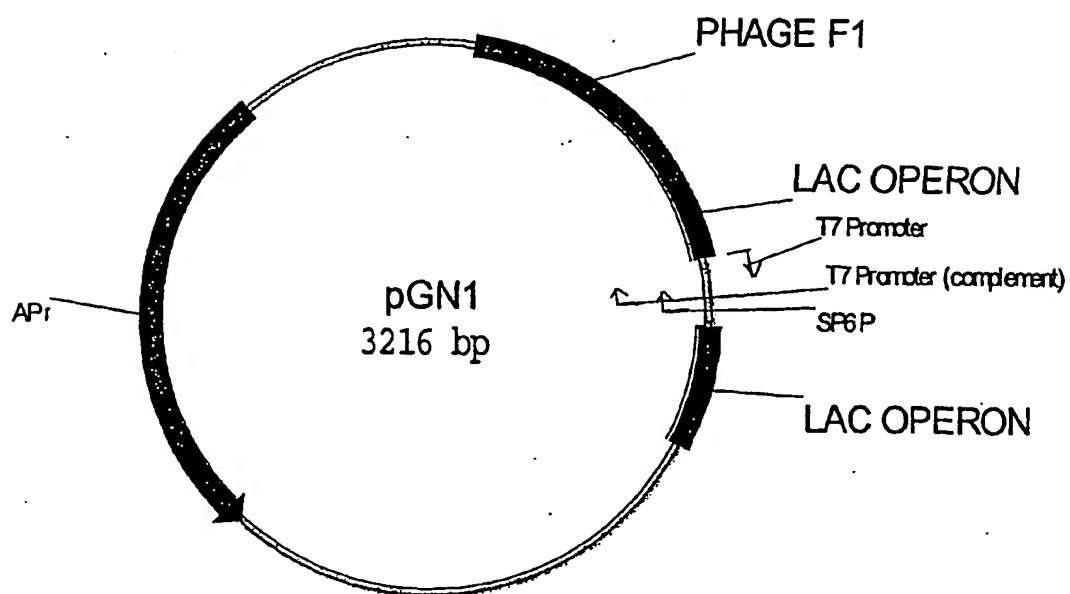
FIG. 1.

FIG. 2.

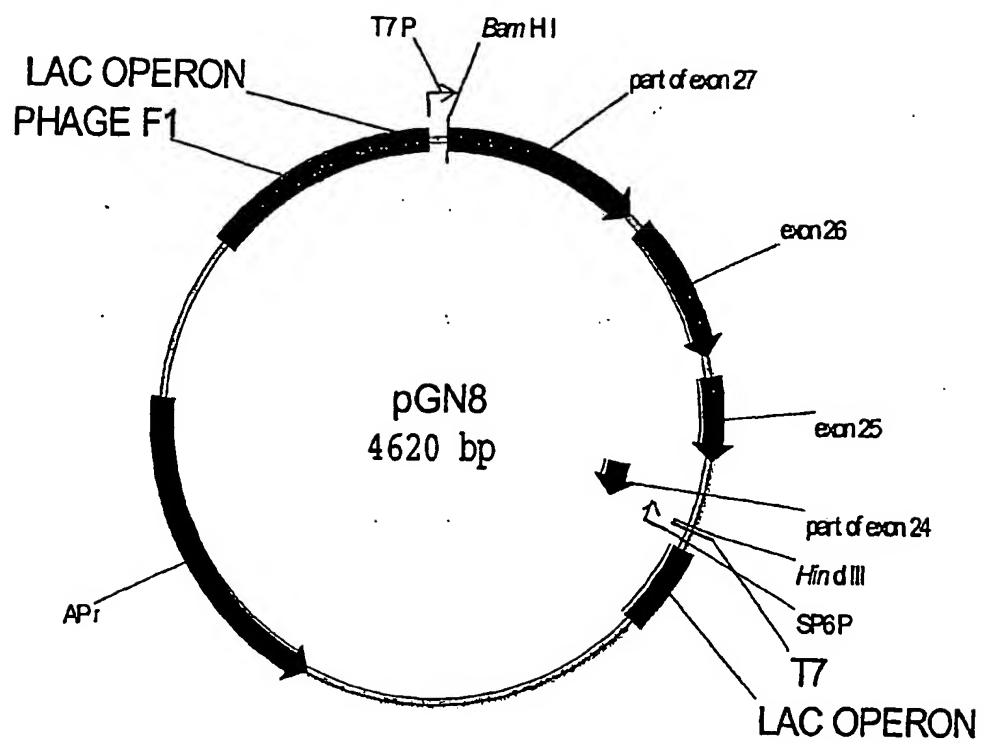


FIG. 3.

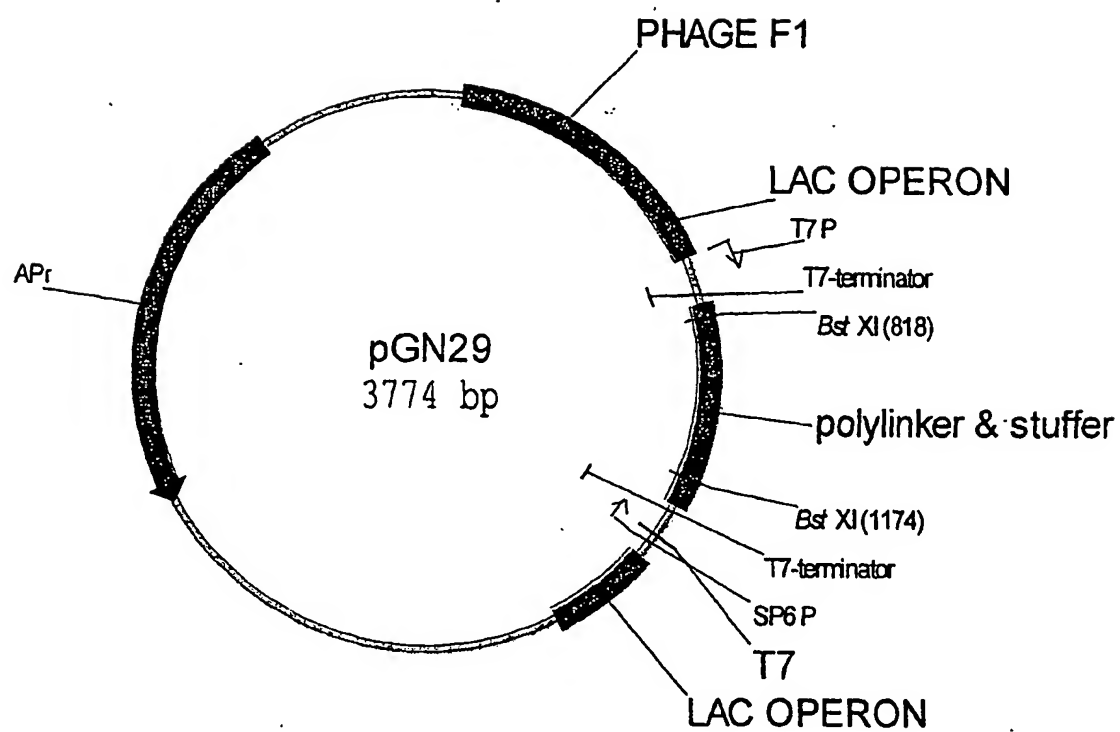


FIG. 4.

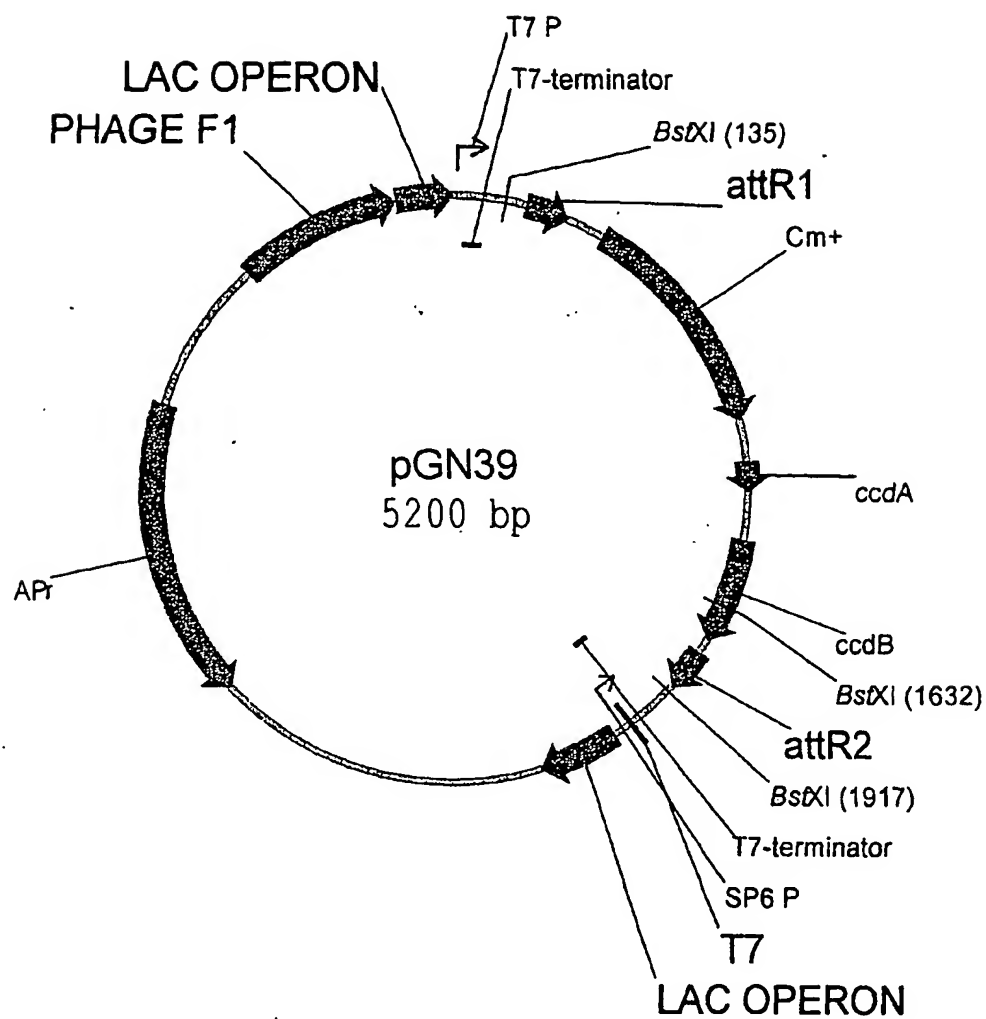


FIG. 5.

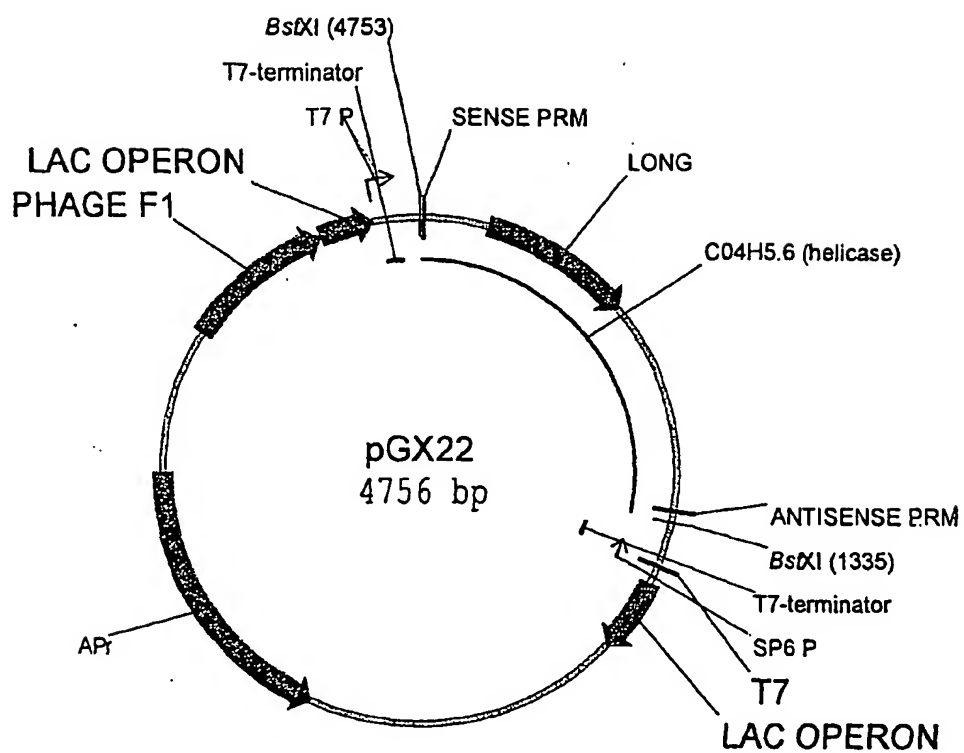


FIG. 6.

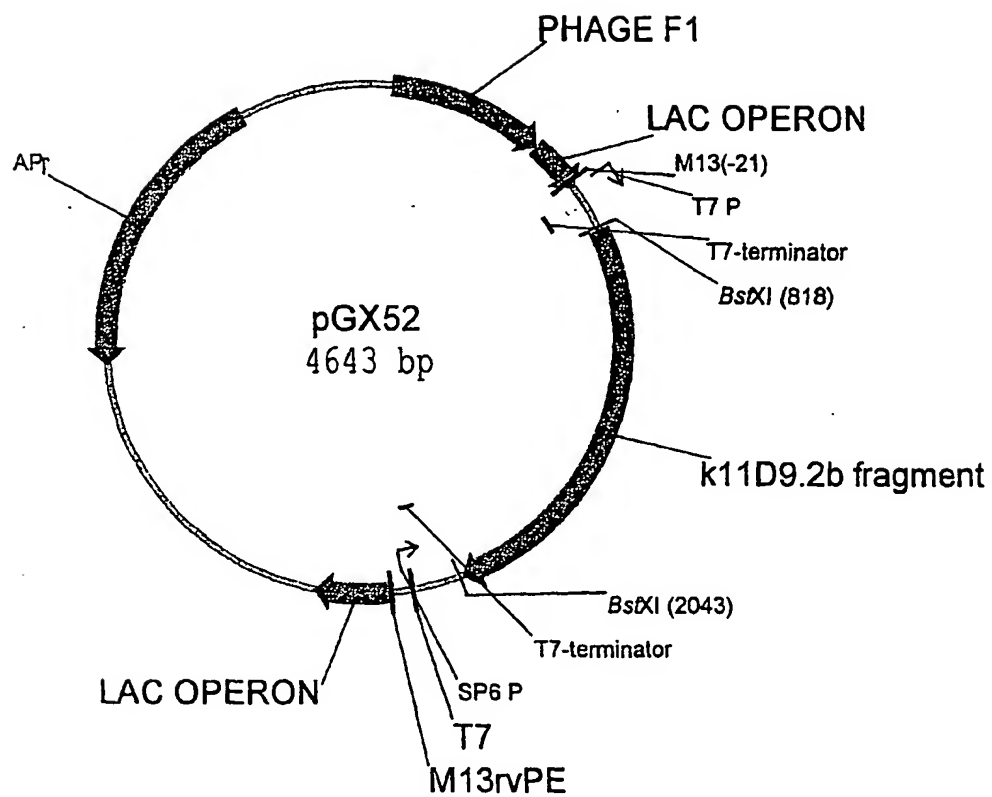


FIG. 7

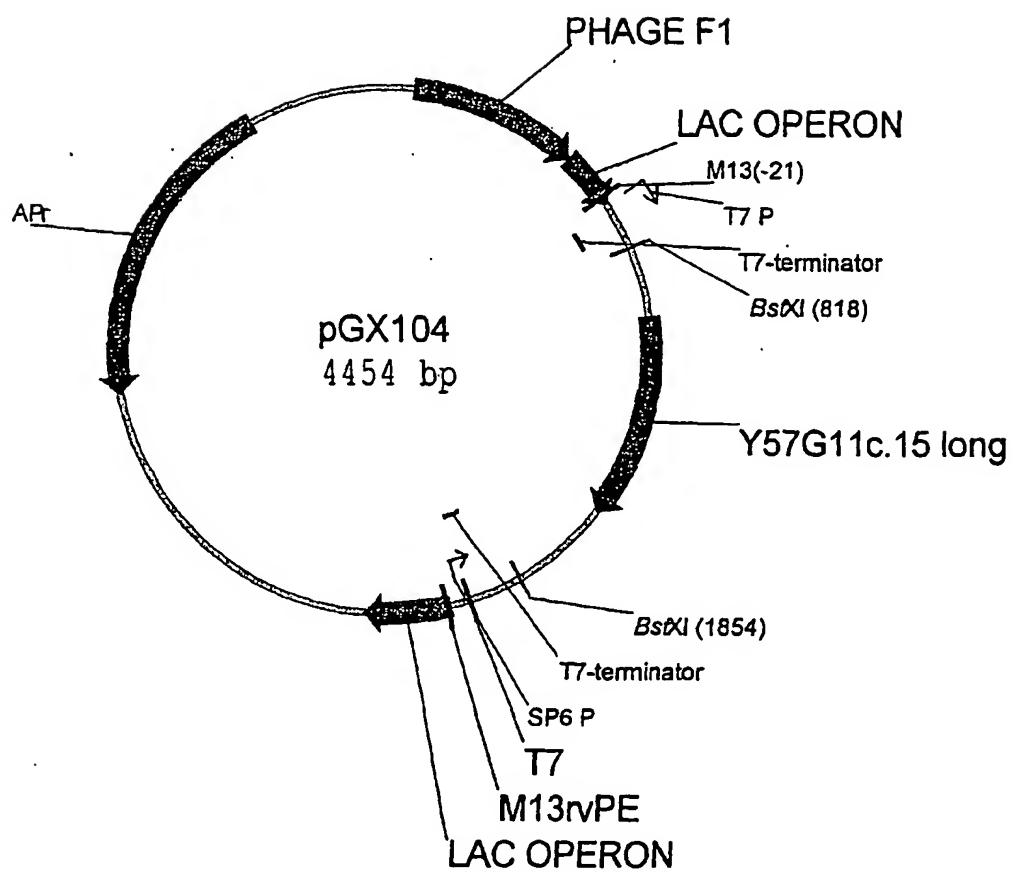


FIG. 8.

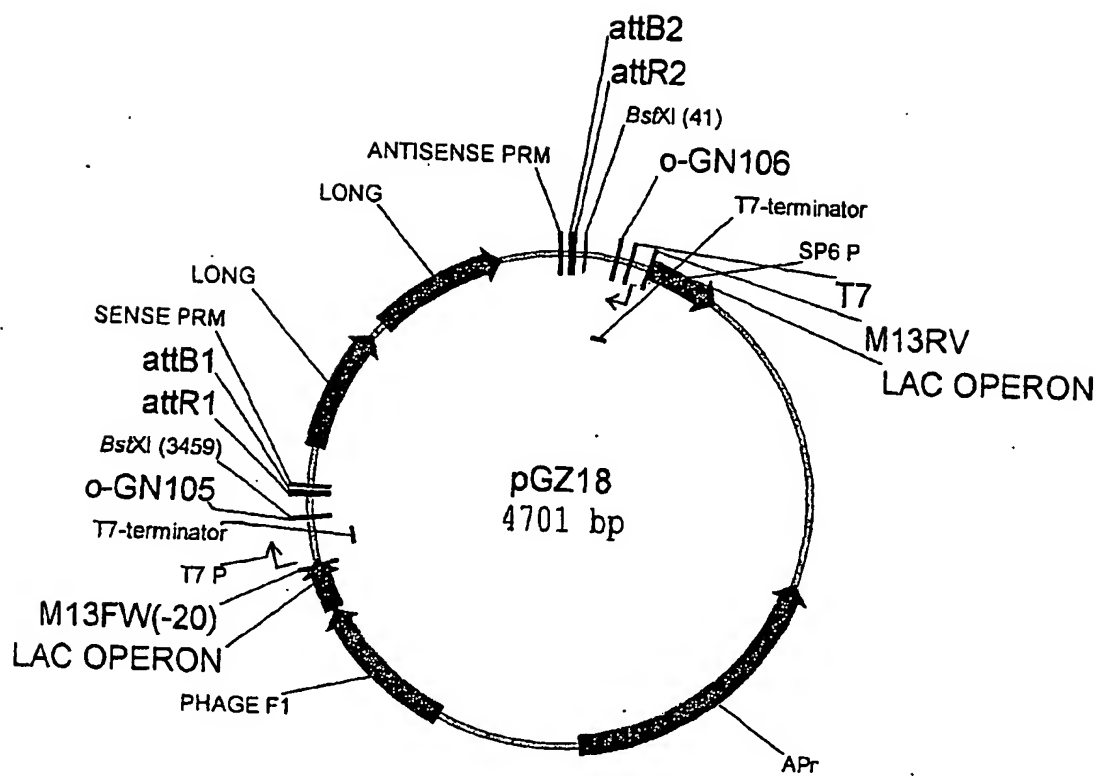


FIG. 9.

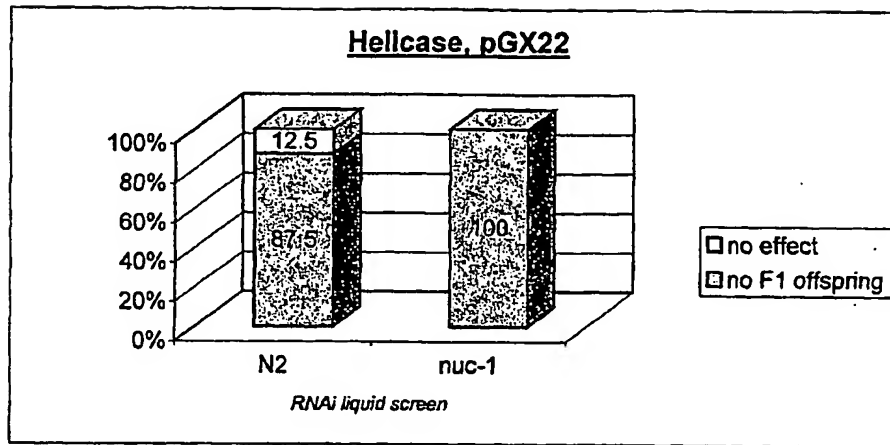


FIG. 10.

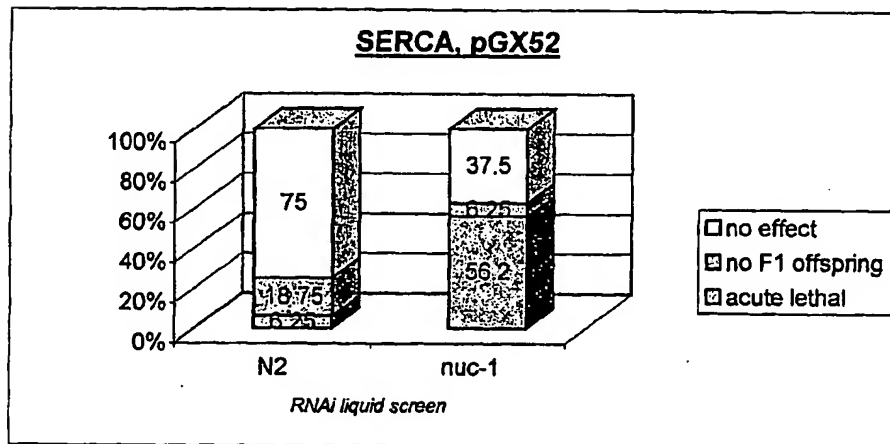


FIG. 11.

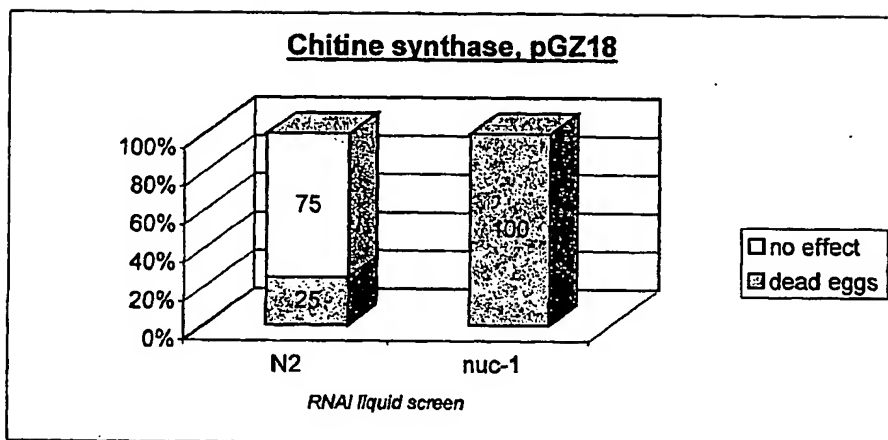
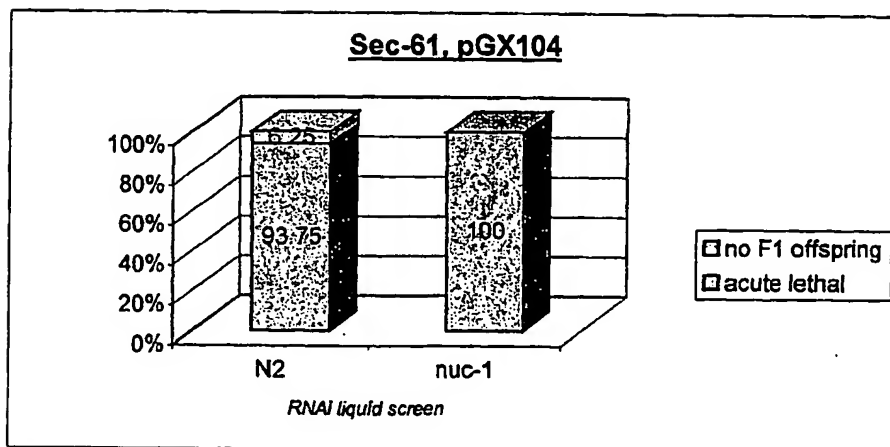


FIG. 12.



SEQUENCE LISTING

<110> DEVGEN NV

<120> IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

<130> SCB/53711/001

<140>

<141>

<160> 14

<170> PatentIn Ver. 2.0

<210> 1

<211> 3216

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN1

<400> 1

```
gagtgcacca tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcatca 60
ggcgaaattg taaacgtaa tattttgtta aaattcgcgt taaatatttg ttaaatacagc 120
tcatttttta accaataggc cgaaatcggc aaaatccctt ataatcaaaa agaataagacc 180
gagatagggt tgagtgttgt tccagtttgg aacaagagtc cactattaaa gaacgtggac 240
tccaacgtca aagggcgaaa aaccgtctat cagggcgatg gccactacg tgaaccatca 300
cccaaatcaa gttttttgcg gtcgaggtgc cgtaaagctc taaatcgga ccctaaagg 360
agccccgat ttagagcttg acggggaaag ccggcgaaag tggcgagaaa ggaagggag 420
aaagcgaaa gaggggcgcg tagggcgctg gcaagtgtag cggtcacgct gcgcgtaacc 480
accacacccg ccgcgcttaa tgcgccgcta cagggcgctg ccattcgcca ttcaggctgc 540
gcaactgttg ggaagggcga tcggtgcggg cctcttcgct attacgccag ctggcgaaag 600
ggggatgtgc tgaagggcga ttaagtggg ttaagccagg gttttcccag tcacgacgtt 660
gtaaaacgac ggccagtga ttgtaatacg actcaactata gggcgaaattc gagctcggta 720
cccggggatc ctctagagtc gaaagcttct cgccctatag tgagtcgtat tacagcttga 780
gtattctata gtgtcaccta aatagcttgg cgtaatcatg gtcatagctg tttcctgtgt 840
gaaattgtta tccgctcaca attccacaca acatacgagc cggaagcata aagtgtaaaag 900
cctgggggtg ctaatgagtg agctaactca cattaattgc gttgcgctca ctgcccgctt 960
tccagtcggg aaacctgtcg tgccagctgc attaatgaat cggccaacgc gcggggagag 1020
gcggttttgc tattgggcgc tcttcgctt cctcgctcac tgactcgctg cgctcggtcg 1080
ttcggtcgc gcgagcggt tcaagtcact caaaggcggg aatacggtta tccacagaat 1140
caggggataa cgcaggaaa aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta 1200
aaaaggccgc gttgctggcg tttttcgata ggctccgccc cctgacgag catcacaaaa 1260
atcgacgctc aagtcagagg tggcgaaacc cgacaggact ataaagatac caggcgtttc 1320
cccctggaag ctccctcgtg cgctctcctg ttccgaccct gccgcttacc ggatacctgt 1380
ccgcctttct cccttcggga agcgtggcgc tttctcatag ctacgctgt aggtatctca 1440
gttcggtgta ggtcgttcgc tccaagctgg gctgtgtgca cgaaccccc gttcagccc 1500
accgctgcgc cttatccggt aactatcgtc ttgagtcctc cccggtaga cagacttat 1560
cgccactggc agcagccact ggtaacagga ttagcagagc gaggtatgta ggcggtgcta 1620
cagagttctt gaagtgttg cctaactacg gctacactag aaggacagta tttggtatct 1680
gcgctctgct gaagccagtt accttcggaa aaagagttgg tagctcttga tccggcaaac 1740
aaaccaccgc tggtagcggg ggtttttttg tttgcaagca gcagattacg cgcagaaaaa 1800
aaggatctca agaagatcct ttgatcttt ctacggggtc tgacgctcag tgaacgaaa 1860
actcacgtta agggattttg gtcatgagat tatcaaaaag gatcttcacc tagatcctt 1920
taaattaaaa atgaagtttt aaatcaatct aaagtatata tgagtaaact tggctcgaca 1980
```

2

```

gttaccaatg cttaatcagt gaggcaccta tctcagcgat ctgtctatctt cgttcatcca 2040
tagttgcctg actccccgtc gtgtagataa ctacgatacg ggagggctta ccatctggcc 2100
ccagtgctgc aatgataccg cgagaccac gctcaccggc tccagattta tcagcaataa 2160
accagccagc cggaagggcc gagcgcagaa gtggctctgc aactttatcc gcctccatcc 2220
agtctattaa ttgttgccgg cattgctaca ggcatcgtgg taagtagttc gccagttaat agtttgcgca 2280
acgtttgttg cttgctaca ggcatcgtgg tgtcacgctc gtcgtttggg atggcttcat 2340
tcagctccgg ttcccaacga tcaaggcgag ttacatgac ccccatgttg tgcaaaaaag 2400
cggttagctc cttcggctct ccgatcgttg tcagaagtaa gttggccgca gtgttatcac 2460
tcatggttat ggcagcactg cataattctc ttactgtcat gccatccgta agatgctttt 2520
ctgtgactgg tgagtactca accaagtcac tctgagaata ccgcgcccgg cgaccgagtt 2580
gctcttgccc ggcgtcaata cgggataata gtgtatgaca tagcagaact ttaaaagtgc 2640
tcattcattg aaaacgttct tcggggcgaa aactctcaag gatcttaccg ctgttgagat 2700
ccagttcgat gtaaccactc cgtgcacca actgatcttc agcatctttt actttcacca 2760
gcgtttctgg gtgagcaaaa acaggaagcg aaaatgccgc aaaaaaggga ataaggcgca 2820
cacggaaatg ttgaatactc atactcttcc tttttcaata ttattgaagc atttatcagg 2880
gttattgtct catgagcgga tacatatttg aatgtattta gaaaaataaa caaatagggg 2940
ttccgcgcac atttccccga aaagtgccac ctgacgtcta agaaaccatt attatcatga 3000
cattaaccta taaaaatagg cgtatcacga ggccctttcg tctcgcgcgt ttcgggtgatg 3060
acggtgaaaa cctctgacac atgcagctcc cggagacggg cacagcttgt ctgtaagcgg 3120
atgccgggag cagacaagcc cgtcagggcg cgtcagcggg tgttgggcggg tgtcggggct 3180
ggcttaacta tgcggcatca gagcagattg tactga 3216

```

<210> 2

<211> 4620

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN8

<400> 2

```

gatccgaatc tccatgtctg ttaacagcct tgacacggaa tttatattca tgcccttgag 60
tcaaactcgtc aacgtggaag ttggtatcct tgctctctcc gcaagcagtc catctgccag 120
tggcagcatc ttgcttttca atgacatagt gactgatttc agctcctcca tcatcttctg 180
gttccctcca tgcagatata acatccatcct tgacaatatt agtgacatcg agagggtccac 240
gtgggcttga ttgatgatca agaacagtaa ccttcaactc agcagtgatc gttccattct 300
cgttctctgc cttgatgata taggttcctg tatccgaacg caaagctctc ttcacatgga 360
atttagtctt gccgtcttca ttgttcaact tcatacgatc atcagattcg actggtgttc 420
cttcgaaagt ccaagtaatt gttggagttg gttcaccact gactggaatg ttcaatgaga 480
agtcttgtcc agccttgacc ttgatttctt gaatcgagtt acgatcgatg actggtggaa 540
ctataattta attcaatgat tattagtaat tgatttagac tcttaccatt tctagccttt 600
gcaacagctg atgctgaatc agatggatct cccaatcctg ccttgttctt ggcacggatt 660
ctgaattcgt actttgatcc ttcccttgaga tttccaacag tagcattcgt ttgtccagct 720
ggaacatgag caacgtcatt ccagaatggc gagaactcgt ccttcatctc aacaacgtat 780
tcctcgattg gagcaccacc gtcgtttgct ggtggcttcc attcaaggtc aacatgatcc 840
ttatcccaat cagtaatttc aggagcattt gtctttcctg gcttgtcaaa tggatctttg 900
gcaagtgtgg ttccgaaggt ctccaatgga tcggactctc cttcagcatt gacggcagcg 960
acacggaact gaaaatcaaa atgtttagg caattgagtt caagattaaa aaattctcac 1020
tttatattca tgtccaggaa taagaccgtc aacaacagct gtagtcttat ctccagcgac 1080
ctttgcagct ggaacccatc ttccacttgc agtatogtac ttttcgatca catagttttc 1140
aattggaata cctccatcat catctggtgc acgccaattc aaagtgacat gatcaccatg 1200
aacatcgga aacatctaag gaccatttgg agaagttggc ttgtctgaaa atttaaaata 1260
taaccaaatt aattgaagaa aactaatgct caccaataac attgatctta acagttgctt 1320
catcttctcc atttgcatg acagctttga tagtgaaagt tccactgtct ccacgttcca 1380
tttgcttca aaccagcttt gattggtatt ctgggttatc aagcttctcg ccctatagtg 1440
agtcgtatta cagcttgagt attctatagt gtcacctaaa tagcttggcg taatcatggg 1500
catagctgtt tcctgtgtga aattgttatc cgtcacacaac tccacacac atacgagccg 1560
gaagcataaa gtgtaaagcc tggggtgcct aatgagtgag ctaactcaca ttaattgcgt 1620

```

tgcgctcact	gcccgccttc	cagtcgggaa	acctgtcgtg	ccagctgcat	taatgaatcg	1680
gccaacgcgc	ggggagaggc	ggtttgcgta	ttgggcgctc	ttccgcttcc	tcgctcactg	1740
actcgctgcg	ctcggtcggt	cggctgcggc	gagcggatc	agctcactca	aaggcggtaa	1800
tacggttatc	cacagaatca	ggggataacg	caggaaagaa	catgtgagca	aaaggccagc	1860
aaaaggccag	gaaccgtaaa	aaggccgcgt	tgctggcggt	tttcgatagg	ctccgcccc	1920
ctgacgagca	tcacaaaaat	cgacgctcaa	gtcagaggtg	gcgaaaccg	acaggactat	1980
aaagatacca	ggcgcttccc	cctggaagct	ccctcgtgcg	ctctcctggt	ccgaccctgc	2040
cgcttaccgg	atacctgtcc	gcctttctcc	cttcgggaag	cgtggcgctt	tctcatagct	2100
cacgctgtag	gtatctcagt	tcggtgtagg	tcgttcgctc	caagctgggc	tgtgtgcacg	2160
aacccccgt	tcagcccgcg	cgctgcgcct	tatccggtaa	ctatcgtctt	gagtccaacc	2220
cggtaagaca	cgacttatcg	ccactggcag	cagccactgg	taacaggatt	agcagagcga	2280
ggtatgtagg	cgggtgctaca	gagttcttga	agtgggtggc	taactacggc	tacactagaa	2340
ggacagtatt	tggtatctgc	gctctgctga	agccagttac	cttcggaaaa	agagttggta	2400
gctcttgatc	cggcaaaaaa	accaccgctg	gtagcgggtg	tttttttggt	tgcaagcagc	2460
agattacgcg	cagaaaaaaa	ggatctcaag	aagatccttt	gatcttttct	acggggtctg	2520
acgctcagtg	gaacgaaaac	tcacgttaag	ggattttggt	catgagatta	tcaaaaagga	2580
tcttcacct	gatcctttta	aattaaaaat	gaagtttta	atcaatctaa	agtatatatg	2640
agtaaacttg	gtctgacagt	taccaatgct	taatcagtg	ggcacctatc	tcagcgatct	2700
gtctatttgc	ttcatccata	gttgccctgac	tccccgtcgt	gtagataact	acgatacggg	2760
agggcttacc	atctggcccc	agtgtgcaa	tgataccgcg	agaccacgc	tcaccggctc	2820
cagattttat	agcaataaac	cagccagccg	gaaggccga	gcgcagaagt	ggctctgcaa	2880
ctttatccgc	ctccatccag	tctattaatt	gttgccggga	agctagagta	agtagttcgc	2940
cagttaatag	tttgcgcaac	gttggtggca	ttgctacagg	catcgtgggtg	tcacgctcgt	3000
cgtttggtat	ggcttcattc	agctccggtt	cccaacgatc	aaggcgagtt	acatgatccc	3060
ccatgttgtg	caaaaaagcg	gttagctcct	tcggtcctcc	gatcgttggtc	agaagtaagt	3120
tggccgcagt	gttatcactc	atggttatgg	cagcactgca	taattctctt	actgtcatgc	3180
catccgtaag	atgcttttct	gtgactggtg	agtactcaac	caagtcattc	tgagaatacc	3240
gcgcccggcg	accgagttgc	tcttgcccgc	cgtcaatacg	ggataatagt	gtatgacata	3300
gcagaacttt	aaaagtgtc	atcattggaa	aacgttcttc	ggggcgaaaa	ctctcaagga	3360
tcttaccgct	gttgagatcc	agttcgatgt	aaccactcgc	tgaccccaac	tgatcttcag	3420
catcttttac	tttcaccagc	gtttctgggt	gagcaaaaac	aggaaggcaa	aatgccgcaa	3480
aaaagggaat	aaggcgacac	cggaaatgtt	gaatactcat	actcttcctt	tttcaatatt	3540
attgaagcat	ttatcagggt	tattgtctca	tgagcggata	catatttgaa	tgtattttaga	3600
aaaataaaca	aatagggtt	ccgcgcacat	ttccccgaaa	agtgccacct	gacgtctaag	3660
aaaccattat	tatcatgaca	ttaacctata	aaaataggcg	tatcacgagg	ccctttcgtc	3720
tcgcgcggtt	cgggtatgac	ggtgaaaacc	tctgacacat	gcagctcccg	gagacggtca	3780
cagcttgtct	gtaagcggat	gccgggagca	gacaagcccg	tcagggcgcg	tcagcgggtg	3840
ttggcgggtg	tcggggctgg	cttaactatg	cggcatcaga	gcagattgta	ctgagagtgc	3900
accatatgcg	gtgtgaaata	ccgcacagat	gcgtaaggag	aaaataaccgc	atcaggcgaa	3960
attgtaaacg	ttaatatttt	gttaaaattc	gcgttaata	tttggttaaat	cagctcattt	4020
tttaaccaat	aggccgaaat	cggcaaaatc	ccttataaat	caaaagaata	gaccgagata	4080
gggttgagtg	ttgttccagt	ttggaacaag	agtccactat	taaagaacgt	ggactccaac	4140
gtcaaagggc	gaaaaaccgt	ctatcagggc	gatggcccac	tacgtgaacc	atcacccaaa	4200
tcaagttttt	tgcggtcgag	gtgccgtaaa	gctctaaatc	ggaaccctaa	agggagcccc	4260
cgatttagag	cttgacgggg	aaagccggcg	aacgtggcga	gaaaggaagg	gaagaaagcg	4320
aaaggagcgg	gcgctagggc	gctggcaagt	gtagcgggtc	cgctgcgcgt	aaccaccaca	4380
cccgcgcgc	ttaatgcgcc	gctacagggc	gcgtccattc	gccattcagg	ctgcgcaact	4440
gttgggaagg	gcgatcgggtg	cgggcctctt	cgctattacg	ccagctggcg	aaaggggat	4500
gtgctgcaag	gcgattaagt	tgggtaacgc	cagggttttc	ccagtcacga	cgttgtaaaa	4560
cgacggccag	tgaattgtaa	tacgactcac	tatagggcga	attcgagctc	ggtaccggg	4620

<210> 3

<211> 4756

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGX22

<400> 3

tgctcagaga	gtttctcaac	gaacccgatt	tggttagtta	taggtaattt	ttagaacatt	60
tacaaaaaca	gcaaaaaaac	caaacattca	ggatttttgt	ttttaattaa	gaaaaaaatc	120
gatcgctctt	aaattttaat	caatacttcg	aataaaccca	aaaaaaaacg	aaaaaaaatc	180
ctgtttccag	tgtaatgatg	attgacgagg	ctcacgaacg	tactctacac	acggatatct	240
tattcgggtt	agtcaaagat	attgcaagat	tccgaaagga	tttgaagctt	ctcatctctt	300
ctgcaacact	tgacgctgaa	aagttctcca	gtttcttcga	cgacgctccg	attttccgaa	360
ttccgggacg	cagattcccc	gtggacattt	actatacaca	ggctcccga	gcggactacg	420
tcgacgcggc	tatcgtcaca	attatgcaga	ttcacttgac	ccagccactt	cccggcgata	480
ttttgggtatt	tctgactggt	caggaagaaa	tcgaaactgt	acaggaagca	cttatggaac	540
ggtcgaaagc	actgggatcg	aagattaagg	agcttattcc	gctgccgggt	tatgcgaatt	600
tgcccagtg	tttgacggcg	aagatttttcg	agccaacgcc	gaaagatgcg	agaaaggtag	660
atttttctta	caaatttttt	ccaaaaaaa	atccgagaaa	aatctacaaa	atttcaggca	720
aaaactggtt	catttttattc	ctaactagtt	ttttagcaaa	cgtttagatt	taacaaaact	780
gaacaaattt	gaagttttcc	aattttaaaaa	ataaatgttt	cggaagttt	attgaaaaat	840
ctgaaattgc	tatctctctg	tatctgcaaa	aaaaacactt	taaaaaatgc	tctgttcttt	900
gaaaatttct	aaactgaaaa	atttgaaatt	tctgaaaatt	gtgataattt	tataaaattt	960
tatagaaaat	gtaagcattc	cagaaaaata	tcaaaaattt	cgagaaaatt	ctgaaaaaat	1020
ccagaaatat	taacagaaaa	aaaatctttt	gaaacatctg	aaaattaaaa	taaattgaat	1080
ttacattttt	ttttttggga	tttctttaa	atcactatga	atttaccact	aaattttttg	1140
caaaaaatta	tttttttaatt	ttcaaaagaa	aagcaagaaa	ttttaaaaata	tcaaaaagtc	1200
caaatttggt	tcggtgaatt	tttaaaataa	cattttcaag	ataattttta	gttaatacaa	1260
acattccacg	cattttctagt	ttcccaaatt	tctctaaatt	tcaggtggtc	ctagcaacta	1320
acattgccag	cacaatggat	ctcgagggat	cttccatacc	taccagttct	gcgcctgcag	1380
gtcgcggccg	cgactctcta	gacgcgtaag	cttactagca	taaccctctg	gggcctctaa	1440
acgggtcttg	aggggttttt	tgagcttctc	gccctatagt	gagtcgtatt	acagcttgag	1500
tattctatag	tgctacctaa	atagcttggc	gtaatcatgg	tcatactgtg	ttcctgtgtg	1560
aaattgttat	ccgctcacaa	ttccacacaa	catcacgagc	ggaagcataa	agtgtaaagc	1620
ctggggtgcc	taatgagtga	gctaactcac	attaattgcy	ttgcgctcac	tgcccgcttt	1680
ccagtcggga	aacctgtcgt	gccagctgca	ttaatgaatc	ggccaacgcg	cggggagagg	1740
cggtttgcgt	attgggcgct	cttccgcttc	ctcgcctact	gactcgctgc	gctcggtcgt	1800
tcggtctgcg	cgagcggtat	cagctcactc	aaaggcggt	atacggttat	ccacagaatc	1860
aggggataac	gcaggaaaaga	acatgtgagc	aaaaggccag	caaaaggcca	ggaaccgtaa	1920
aaaggccgcg	ttgtctggcg	ttttcgatag	gtcccgcccc	cctgacgagc	atcacaaaaa	1980
tcgacgctca	agccagttta	ggcgaaaccc	catcaggacta	taaagatacc	aggcgtttcc	2040
ccctggaagc	tccctcgtgc	gctctcctgt	tccgaccctg	ccgcttaccg	gatacctgtc	2100
cgcttttctc	ccttcgggaa	gcgtggcgct	ttctcatagc	tcacgctgta	ggtatctcag	2160
ttcggtgtag	gtcggttcgct	ccaagctggg	ctgtgtgcac	gaaccccccg	ttcagcccga	2220
ccgctgcgcc	ttatccggta	actatcgtct	tgagtccaac	ccggtaagac	acgacttatc	2280
gccactggca	gcagccactg	gtaacaggat	tagcagagcg	aggtatgtag	gcgggtgctac	2340
agagttcttg	aagtgggtgc	ctaactacgg	ctacactaga	aggacagtat	ttggtatctg	2400
cgctctgctg	aagccagtta	ccttcggaaa	aagagtgggt	agctcttgat	ccggcaaaaa	2460
aaccaccgct	ggtagcgggtg	gtttttttgt	ttgcaagcag	cagattacgc	gcagaaaaaa	2520
aggatctcaa	gaagatcctt	tgatcttttc	tacgggggtct	gacgctcagt	ggaacgaaaa	2580
ctcacgttaa	gggatttttg	tcatgagatt	atcaaaaagg	atcttcacct	agatcctttt	2640
aaattaaaaa	tgaagtttta	aatcaatcta	aagtatatat	gagtaaactt	ggtctgacag	2700
ttaccaatgc	ttaatcagtg	aggcacctat	ctcagcgatc	tgtctatttc	gttcatccat	2760
agttgcctga	ctccccgtcg	tgtagataac	tacgatacgg	gagggtttac	catctggccc	2820
cagtgtcgca	atgataccgc	gagaccacg	ctcaccggct	ccagatttat	cagcaataaa	2880
ccagccagcc	ggaagggccg	agcgcagaag	tggtcctgca	actttatccg	cctccatcca	2940
gtctattaat	tggtgccggg	aagctagagt	aagtagttcg	ccagtttaata	gtttgcgcaa	3000
cgttgttggc	attgctacag	gcacgtgggt	gtcacgctcg	tcgtttggta	tggtttcatt	3060
cagctccggg	tcccaacgat	caaggcgagt	tacatgatcc	cccatgttgt	gcaaaaaagc	3120
ggttagctcc	ttcggtcctc	cgatcgttgt	cagaagtaag	ttggccgcag	tggttatcact	3180
catggttatg	gcagcactgc	ataattctct	tactgtcatg	ccatccgtaa	gatgcttttc	3240
tgtgactggg	gagtactcaa	ccaagtcatt	ctgagaatac	cgcgccgggc	gaccgagttg	3300
ctcttgcggc	gcgtcaatac	gggataatag	tgtatgacat	agcagaactt	taaaagtgc	3360

catcattgga	aaacgttctt	cggggcgaaa	actctcaagg	atcttaccgc	tgttgagatc	3420
cagttcga	taaccactc	gtgcacccaa	ctgatcttca	gcatctttta	ctttcaccag	3480
cgtttctggg	tgagcaaaaa	caggaaggca	aaatgccgca	aaaaagggaa	taagggcgac	3540
acggaaatgt	tgaatactca	tactcttcct	ttttcaatat	tattgaagca	tttatcaggg	3600
ttattgtctc	atgagcggat	acatatttga	atgtatttag	aaaaataaac	aaataggggt	3660
tccgcgcaca	tttccccgaa	aagtgccacc	tgacgtctaa	gaaaccatta	ttatcatgac	3720
attaacctat	aaaaataggc	gtatcacgag	gccctttcgt	ctcgcgcgtt	tcggtgatga	3780
cggtgacaaa	ctctgacaca	tgacgtccc	ggagacggtc	acagcttgtc	tgtaagcgga	3840
tgccggggagc	agacaagccc	gtcagggcgc	gtcagcgggt	gttggcgggt	gtcggggctg	3900
gcttaactat	gcggcatcag	agcagattgt	actgagagtg	caccatatgc	ggtgtgaaat	3960
accgcacaga	tgcgtaagga	gaaaataccg	catcaggcga	aattgtaaac	gttaatat	4020
tggtaaaaat	cgcggttaaat	atttggttaa	tcagctcatt	ttttaaccaa	taggccgaaa	4080
tcggcaaaaat	cccttataaa	tcaaaagaat	agaccgagat	agggttgagt	ggtgttcag	4140
tttggaacaa	gagtccacta	ttaaagaacg	tggactccaa	cgtaaaagg	cgaaaaaccg	4200
ctatcacagg	cgatggccca	ctacgtgaac	catcacccaa	atcaagtttt	ttgcggtcga	4260
ggtgccgtaa	agctctaaat	cggaaacccta	aagggaagccc	ccgatttaga	gcttgacggg	4320
gaaagccggc	gaacgtggcg	agaaaggaag	ggaagaaagc	gaaaggagcg	ggcgctaggg	4380
cgctggcaag	tgtagcggtc	acgctgcgcg	taaccaccac	acccgccgcg	cttaatgcgc	4440
cgctacaggg	cgcgctccatt	cgccattcag	gctgcgcaac	tggtgggaag	ggcgatcgg	4500
gcgggcctct	tcgctattac	gccagctggc	gaaaggggga	tggtctgcaa	ggcgattaag	4560
ttgggtaaog	ccagggtttt	cccagtcacg	acgttgtaaa	acgacggcca	gtgaattgta	4620
atacgactca	ctatagggcg	aattcaaaaa	acccctcaag	acccgtttag	aggccccaag	4680
gggttatgct	agtgaattct	gcagggtacc	cggggatcct	ctagagatcc	ctcgacctcg	4740
agatccattg	tgctgg					4756

<210> 4

<211> 4643

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGX52

<400> 4

gagtgacaca	tatgcgggtgt	gaaataccgc	acagatgcgt	aaggagaaaa	taccgcatca	60
ggcgaaattg	taaacgttaa	tattttgtta	aaatccgcgt	taaatatttg	ttaaatcagc	120
tcatttttta	accaataggc	cgaaatccgc	aaaatccctt	ataaatcaaa	agaatagacc	180
gagatagggt	tgagtgttgt	tccagtttgg	aacaagagtc	cactattaaa	gaacgtggac	240
tccaacgtca	aagggcgaaa	aaccgtctat	cagggcgatg	gcccactacg	tgaaccatca	300
cccaaatcaa	gttttttgcg	gtcgaggtgc	cgtaaaagtc	taaatacgaa	ccctaaagg	360
agcccccgat	ttagagcttg	acggggaaa	ccggcgaaag	tgccgagaaa	ggaagggaag	420
aaagcgaaa	gagcgggcgc	tagggcgctg	gcaagtgtag	cggtcacgct	gcgcgtaacc	480
accacaccog	ccgcgcttaa	tgcccgcta	cagggcgcg	ccattcgcca	ttcaggctgc	540
gcaactgttg	ggaagggcga	tcggtgcggg	cctcttcgct	attacgccag	ctggcgaaag	600
ggggatgtgc	tgcaaggcga	ttagttggg	taacgccagg	gttttccag	tcacgacgtt	660
gtaaaacgac	ggccagtga	ttgtaatac	actcactata	ggcggaattc	aaaaaacccc	720
tcaagaccog	tttagaggcc	ccaaggggtt	atgctagtga	attctgcagg	gtaccgggg	780
atcctctaga	gatccctcga	cctcgagatc	cattgtgtctg	gcagccgatc	tccgtcttgt	840
gaagatctac	tccaccacca	tccgtatcga	tcagtccatc	ctcaccggag	aatctgtgtc	900
tgttatcaag	cacaccgact	ctgtgccaga	tccacgcgct	gttaaccagg	acaagaagaa	960
ttgtctgttc	tcgggaacca	atgtcgcatc	tggaaaggct	cgtggaatcg	tcttcggaac	1020
cggattgacc	actgaaatcg	gaaagatccg	taccgaaatg	gctgagaccg	agaatgagaa	1080
gacaccactt	caacagaagt	tggacgaatt	cggagagcaa	ctttccaagg	ttatctctgt	1140
tatttgogtt	gctgtttggg	ctatcaacat	tggacatttc	aacgatccag	ctcacggtgg	1200
atcatggggt	aagggagcaa	tctactactt	caaaatcgcc	gttgctcttg	ccgtcgctgc	1260
tattccagaa	ggacttccag	ctgtcatcac	cacgtgcctt	gccctcgaa	ctcgccgtat	1320
ggccaagaag	aacgctattg	taagatccct	tccatccgct	gaaactcttg	gatgcacatc	1380
tgttatctgc	tctgacaaga	ctggaactct	caccaccaac	cagatgtctg	tgtcaaagat	1440

gttcacgcgt	ggacaagcct	ctggagacaa	catcaacttc	accgagttcg	ccatctccgg	1500
atccacctac	gagccagtcg	gaaagggttc	caccaatgga	cgtgaaatca	acccagctgc	1560
tggagaattc	gaatcactca	ccgagttggc	catgatctgc	gctatgtgca	atgattcatc	1620
tgttgattac	aatgagacca	agaagatcta	cgagaaagtc	ggagaagcca	ctgaaactgc	1680
tcttatcggt	cttgctgaga	agatgaatgt	tttcggaacc	tcgaaagccg	gactttcacc	1740
aaaggagctc	ggaggagttt	gcaaccgtgt	catccaacaa	aaatggaaga	aggagttcac	1800
actcgagttc	tcccgtgatc	gtaaattccat	gtccgcctac	tgcttcccag	cttccggagg	1860
atctggagcc	aagatgttcg	tgaaggagc	cccagaagga	gttctcggaa	gatgcacca	1920
cgtcagagtt	aacggacaaa	aggttccact	cacctctgcc	atgactcaga	agattgttga	1980
ccaatgcgtg	caatacggaa	ccggaagaga	tacccttcgt	tgtcttgccc	tcggccagca	2040
caatggatct	cgagggatct	tccataccta	ccagttctgc	gcctgcaggt	cgcgcccgcg	2100
actctctaga	cgcgtaagct	tactagcata	accccttggg	gcctctaaac	gggtcttgag	2160
gggttttttg	agcttctcgc	cctatagtga	gtcgtattac	agcttgagta	ttctatagt	2220
tcacctaaat	agcttggcgt	aatcatggtc	atagctgttt	cctgtgtgaa	attgttatcc	2280
gtcaccaatt	ccacacaaca	tacgagccgg	aagcataaag	tgtaaagcct	ggggtgccta	2340
atgagtgagc	taactcacat	taattgcgtt	gcgtcactg	cccgccttcc	agtcgggaaa	2400
cctgtcgtgc	cagctgcatt	aatgaatcgg	ccaacgcgcg	gggagaggcg	gtttgcgtat	2460
tgggcgctct	tccgcttcct	cgctcactga	ctcgctgcgc	tcggtcgttc	ggctgcggcg	2520
agcggtatca	gctcactcaa	aggcggtaat	acggttatcc	acagaatcag	gggataacgc	2580
aggaaagaac	atgtgagcaa	aaggccagca	aaaggccagg	aaccgtaaaa	aggccgcgtt	2640
gctggcggtt	ttcgataggc	tccgcccccc	tgacgagcat	cacaaaaatc	gacgctcaag	2700
tcagaggttg	cgaaccgga	caggactata	aagataccag	gcgtttcccc	ctggaagctc	2760
cctcgtgcgc	tctcctgttc	cgaccctgcc	gcttacggga	tacctgtccg	cctttctccc	2820
ttcggaagc	gtggcgcttt	ctcatagctc	acgctgtagg	tatctcagtt	cggtgtaggt	2880
cgttcgctcc	aagctgggct	gtgtgcacga	acccccggtt	cagcccgacc	gctgcgcctt	2940
atccggtaac	tatcgtcttg	agtccaaccc	ggtaagacac	gacttatcgc	cactggcagc	3000
agccactggt	aacaggatta	gcagagcgag	gtatgtaggc	ggtgctacag	agttcttgaa	3060
gtggtggcct	aactacggct	acactagaag	gacagtattt	ggtatctgcg	ctctgctgaa	3120
gccagttacc	ttcggaaaaa	gagttggtag	ctcttgatcc	ggcaaaaaaa	ccaccgctgg	3180
tagcgtgggt	tttttgtttt	gcaagcagca	gattacgcgc	agaaaaaaaag	gatctcaaga	3240
agatcctttg	atcttttcta	cggggtctga	cgctcagtg	aacgaaaact	cacgttaagg	3300
gatttttggtc	atgagattat	caaaaaggat	cttcacctag	atccttttaa	attaaaaatg	3360
aagttttaaa	tcaatctaaa	gtatatatga	gtaaacttgg	tctgacagtt	accaatgctt	3420
aatcagtgag	gcacctatct	cagcgatctg	tctatttcgt	tcatccatag	ttgcctgact	3480
ccccgtcgtg	tagataacta	cgatacggga	gggcttacca	tctggcccca	gtgctgcaat	3540
gataccgctg	gaccacgct	caccggctcc	agatttatca	gcaataaacc	agccagccgg	3600
aagggccgag	cgcagaagtg	gtcctgcaac	tttatccgcc	tccatccagt	ctattaattg	3660
ttgccgggaa	gctagagtaa	gtagttcgcc	agttaatagt	ttgcgcaacg	ttgttggcat	3720
tgctacaggc	atcgtgggtg	cacgctcgtc	gtttggtagt	gcttcattca	gctccggttc	3780
ccaacgatca	aggcgagtta	catgatcccc	catgttgtgc	aaaaaagcgg	ttagctcctt	3840
cggctcctccg	atcgttgtca	gaagtaagtt	ggccgcagtg	ttatcactca	tggttatggc	3900
agcactgcat	aattctctta	ctgtcatgcc	atccgtaaga	tgcttttctg	tgactggtga	3960
gtactcaacc	aagtcattct	gagaataccg	cgcgcggcga	ccgagttgct	cttgcccgccg	4020
gtcaatacgg	gataatagtg	tatgacatag	cagaacttta	aaagtgtctca	tcattggaaa	4080
acgttcttcg	gggcgaaaaac	tctcaaggat	cttacccgtg	ttgagatcca	gttcgatgta	4140
accactcgt	gcacccaact	gatcttcagc	atcttttact	ttcaccagcg	tttctgggtg	4200
agcaaaaaaca	ggaaggcaaa	atgccgcaaa	aaagggaata	agggcgacac	ggaaatgttg	4260
aatactcata	ctcttccttt	ttcaatatta	ttgaagcatt	tatcagggtt	attgtctcat	4320
gagcggatac	atatttgaat	gtatttagaa	aaataaacia	ataggggttc	cgcgcacatt	4380
tccccgaaaa	gtgccacctg	acgtctaaga	aaccattatt	atcatgacat	taacctataa	4440
aataggcgt	atcacgaggc	cctttcgtct	cgcgcgtttc	ggtgatgacg	gtgaaaacct	4500
ctgacacatg	cagctcccg	agacggtcac	agcttgtctg	taagcggatg	ccgggagcag	4560
acaagcccgt	cagggcgcgt	cagcgggtgt	tggcgggtgt	cggggctggc	ttaactatgc	4620
ggcatcagag	cagattgtac	tga				4643

<210> 5

<211> 4454

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGX104

<400> 5

gagtgaccca	tatgcggtgt	gaaataccgc	acagatgcgt	aaggagaaaa	taccgcatca	60
ggcgaaattg	taaacgttaa	tattttgtta	aaattcgcgt	taaatatttg	ttaaatcagc	120
tcatttttta	accaataggc	cgaaatcggc	aaaatccctt	ataaatcaaa	agaatagacc	180
gagatagggt	tgagtgttgt	tccagtttgg	aacaagagtc	cactattaaa	gaacgtggac	240
tccaacgtca	aagggcgaaa	aaccgtctat	cagggcgatg	gcccactacg	tgaaccatca	300
cccaaatcaa	gttttttgcg	gtcagaggtg	cgtaaagctc	taaatcggaa	ccctaaaggg	360
agccccgat	ttagagcttg	acggggaaa	cgggcgaacg	tggcgagaaa	ggaagggaag	420
aaagcgaaag	gagcgggctg	tagggcgctg	gcaagtgtag	cggtcacgct	gcgcgtaacc	480
accacaccgc	ccgcgcttaa	tgcgcgcta	cagggcgctg	ccattcgcca	ttcaggctgc	540
gcaactgttg	ggaagggcga	tcggtgcggg	cctcttcgct	attacgccag	ctggcgaaa	600
ggggatgtgc	tgcaaggcga	ttaagttggg	taacgccagg	gttttcccag	tcacgacgtt	660
gtaaaacgac	ggccagtga	ttgtaatacg	actcactata	gggcgaattc	aaaaaacccc	720
tcaagaccgc	tttagaggcc	ccaaggggtt	atgctagtga	attctgcagg	gtaccggggg	780
atcctctaga	gatccctcga	cctcgagatc	cattgtgctg	gaccgtggta	ctcttatgga	840
gctcggaatc	tcgccaatcg	tcacttctgg	acttatcatg	caacttctcg	ccggagccaa	900
gatcatcgaa	gtcggagaca	caccaaagga	ccgtgctctt	ttcaacggag	ccagaaatg	960
taagccgaaa	agtgtgtgtt	ttcaatctct	aatttttgaa	cttttcagtg	ttcgggtatg	1020
tcactactgt	tggacaagct	attgtctacg	tcattgtccg	actctacgga	gagccatcgg	1080
aaatcggagc	tggaatctgt	ctccttatcg	tcgtccaact	cggtattgcc	ggtctcatcg	1140
tcctccttct	cgacgagctt	ctccaaaagg	gatatggtct	cggatccgga	atttctctct	1200
tcattgccac	caacatctgt	gaaaccattg	tctggaaggc	attctccccg	gcaacaatga	1260
acaccggacg	tggaaccgag	ttcgaaggag	ccgtcattgc	tcttttccat	cttcttgcca	1320
cccgctccga	caaggtccgt	gcccttcgtg	aggctttcta	ccgtcaaaac	cttccaaact	1380
tgatgaactt	gattggtaact	ttcctcgttt	tgcgggtggt	tatctacttc	caaggattcc	1440
gtgtcgacct	cccaatcaag	tctgcccgtc	accgtggaca	atacagcagc	taccaaatca	1500
agctcttcta	cacctccaac	attccaatca	tccttcaatc	tgctctcgtc	tccaacctct	1560
acgttatctc	tcaggtttgt	tgcattctcag	tagtacgggt	agatgtttat	ctttctctag	1620
agggccaagt	tgcccgagaa	atttttttgag	ttcattctca	agtctgatgg	aaaatgttta	1680
tttttcagat	gctcgccgga	aagttcggag	gaaacttctt	catcaacctt	ctcgggtacct	1740
ggtccgataa	cacgggatac	agaagctacc	acactggagg	actctgctac	tatctttcac	1800
caccagagtc	tcttgagcac	atcttcgaag	acccaatcca	ctgcaccagc	acaatggatc	1860
tcgagggatc	ttccatacct	accagttctg	cgcttcgagg	tcgcggccgc	gactctctag	1920
acgcgtaagc	ttactagcat	aaccctcttg	ggcctctaaa	cggtctctga	ggggtttttt	1980
gagcttctcg	ccctatagtg	agtcgtatta	cagcttgagt	attctatagt	gtcacctaaa	2040
tagcttggcg	taatcatggt	catagctgtt	tcctgtgtga	aattgttatc	cgctcacaat	2100
tccacacaac	atacagagcg	gaagcataaa	gtgtaaagcc	tgggtgacct	aatgagttag	2160
ctaactcaca	ttaattgcgt	tgcgctcact	gccgcttttc	cagtcgggaa	acctgtcgtg	2220
ccagctgcat	taatgaatcg	gccaacgcgc	ggggagaggc	ggtttgcgta	ttgggcgctc	2280
ttccgcttcc	tcgctcactg	actcgtcgcg	ctcggtcggt	cggtgcgggc	gagcgggtatc	2340
agctcactca	aaggcggtaa	tacggttatc	cacagaatca	ggggataacg	caggaaagaa	2400
catgtgagca	aaaggccagc	aaaaggccag	gaaccgtaaa	aaggccgcgt	tgctggcggt	2460
tttcgatagg	ctccgcccc	ctgacgagca	tcacaaaaat	cgacgctcaa	gtcagaggtg	2520
gcgaaacccg	acaggactat	aaagatacca	ggcgtttccc	cctggaagct	ccctcgtgcg	2580
ctctcctggt	ccgacctgct	cgcttaccgg	atacctgtcc	gcctttctcc	cttcgggaag	2640
cgtaggcgtt	tctcatagct	cacgctgtag	gtatctcagt	tcggtgtagg	tcgttcgctc	2700
caagctgggc	tgtgtgcacg	aacccccgtt	tcagcccgac	cgctgcgcct	tatccggtaa	2760
ctatcgtctt	gagtccaacc	cggttaagaca	cgacttatcg	ccactggcag	cagccactgg	2820
taacaggatt	agcagagcga	ggtatgtagg	cggtgctaca	gagttcttga	agtgggtggc	2880
taactacggc	tacactagaa	ggacagtatt	tggtatctgc	gctctgctga	agccagttac	2940
cttcggaaaa	agagttggta	gctcttgatc	cggcaaaaaa	accaccgctg	gtagcgggtg	3000
ttttttgtt	tgcaagcagc	agattacgcg	cagaaaaaaa	ggatctcaag	aagatccttt	3060
gatcttttct	acggggctcg	acgctcagtg	gaacgaaaaa	tcacgttaag	ggattttggt	3120

```

catgagatta tcaaaaagga tcttcaccta gatcctttta aattaaataat gaagttttta 3180
atcaatctaa agtatatatg agtaaacttg gtctgacagt taccaatgct taatcagtga 3240
ggcacctatc tcagcgatct gtctatttcg ttcattccata gttgcctgac tccccgtcgt 3300
gtagataaact acgatacggg agggcttacc atctggcccc agtgctgcaa tgataccgcg 3360
agaccacgc tcaccggctc cagattttatc agcaataaac cagccagccg gaagggccga 3420
gcgcagaagt ggtcctgcaa ctttatccgc ctccatccag tctattaatt gttgccggga 3480
agctagagta agtagttcgc cagttaatag tttgcgcaac gttgttgga ttgctacagg 3540
catcgtgggtg tcacgctcgt cgtttgggtat ggcttcattc agctccggtt cccaacgac 3600
aaggcgagtt acatgatccc ccatgtttgtg caaaaaagcg gttagctcct tcggtcctcc 3660
gatcgtttgc agaagtaagt tggccgcagt gttatcactc atggttatgg cagcactgca 3720
taattctctt actgtcatgc catccgtaag atgcttttct gtgactggtg agtactcaac 3780
caagtcattc tgagaatacc gcgcccggcg accgagttgc tcttgcccg cgtcaatacg 3840
ggataatagt gtatgacata gcagaacttt aaaagtgtc atcattggaa aacgttcttc 3900
ggggcgaaaa ctctcaagga tcttaccgct gttgagatcc agttcgatgt aaccactcg 3960
tgacaccaac tatgtttcag catcttttct gttcaccagc gtttctgggt gagcaaaaac 4020
aggaagcaca aatgccgcaa aaaagggaat aaggcgaca cggaaatgtt gaatactcat 4080
actcttctt tttcaatatt attgaagcat ttatcagggt tattgtctca tgagcgga 4140
catatttgaa tgtattttaga aaaataaaca aataggggtt ccgcgcacat tccccgaaa 4200
agtgccacct gacgtctaag aaaccattat tatcatgaca ttaacctata aaaataggcg 4260
tatcacgagg ccctttcgtc tcgcgcgttt cggtagtgac ggtgaaaacc tctgacacat 4320
gcagctcccg gagacggtca cagcttgtct gtaagcggat gccgggagca gacaagcccg 4380
tcagggcgcg tcagcgggtg ttggcgggtg tcggggctgg cttaactatg cggcatcaga 4440
gcagattgta ctga 4454

```

<210> 6

<211> 4701

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGZ18

<400> 6

```

accagcttt cttgtacaaa gtggtgatct ttccagcaca atggatctcg agggatcttc 60
catacctacc agttctgcgc ctgcaggctc cggccgcgac tctctagacg cgtaagctta 120
ctagcataac cccttggggc ctctaaacgg gtcttgaggg gttttttgag cttctcgccc 180
tatagtgagt cgtattacag cttgagtatt ctatagtgtc acctaaatag cttggcgtaa 240
tcatggcat agctgtttcc tgtgtgaaat tgttatccgc tcacaattcc acacaacata 300
cgagccgga gcataaagtg taaagcctgg ggtgcctaata gaggtagcta actcacatta 360
attgcgttgc gctcactgcc cgctttccag tcgggaaacc tgcgtgccca gctgcattaa 420
tgaatcggcc aacgcgcggg gagaggcgggt ttgctatttg ggctctcttc cgttcctctg 480
ctcactgact cgctgcgctc ggtcgttcgg ctgcggcgag cggtatcagc tcaactcaaag 540
gcgtaatac ggttatccac agaatacagg gataacgcag gaaagaacat gtgagcaaaa 600
ggccagcaaa aggccaggaa ccgtaaaaag gccgcgttgc tggcgttttt cgataggctc 660
cgccccctg acgagcatca caaaaatcga cgctcaagtc agaggtggcg aaaccgcaca 720
ggactataaa gataccaggc gtttccccct ggaagctccc tcgtgcgctc tctgtttccg 780
accctgccgc ttaccggata cctgtccgcc tttctccctt cgggaagcgt ggcgctttct 840
catagctcac gctgtaggta tctcagttcg gtgtaggctg ttcgctccaa gctgggctgt 900
gtgcacgaac cccccgttca gccgcaccgc tgcgccttat ccggtacta tcgtcttgag 960
tccaacccgg taagacacga cttatcgcca ctggcagcag ccactggtta caggattagc 1020
agagcgaggt atgtaggcgg tgctacagag ttcttgaaat ggtggcctaa ctacggctac 1080
actagaagga cagtatttgg tatctgcgct ctgctgaagc cagttacctt cggaaaaaga 1140
gttggttagc cttgatccgg caaacaacc accgctggta gcggtgggtt ttttgtttgc 1200
aagcagcaga ttacgcgcag aaaaaaagga tctcaagaag atcctttgat cttttctacg 1260
gggtctgacg ctcagtggaa cgaaaactca cgtaaggga ttttggctat gagattatca 1320
aaaaggatct tcacctagat ccttttaaat taaaaatgaa gttttaaatc aatctaaagt 1380
atatatgagt aaacttggtc tgacagttac caatgcttaa tcagtggagc acctatctca 1440
gcgactctgc tatttctgtc atccatagtt gcctgactcc ccgtcgtgta gataactacg 1500

```

atacgggagg	gcttaccatc	tggccccagt	gctgcaatga	taccgcgaga	cccacgctca	1560
ccggctccag	atztatcagc	aataaaccag	ccagccggaa	gggcccagcg	cagaagtggg	1620
cctgcaactt	tatccgcctc	catccagttc	attaattggt	gccgggaagc	tagagtaagt	1680
agttcgccag	ttaatagttt	gcgcaacggt	gttggcattg	ctacaggcat	cgtgggtgtca	1740
cgctcgctcg	ttggtatggc	ttcattcagc	tccggttccc	aacgatcaag	gcgagttaca	1800
tgatccccc	tggtgtgcaa	aaaagcggtt	agtccttcg	gtcctccgat	cgttgtcaga	1860
agtaagtgg	ccgcagtgtt	atcactcatg	gttatggcag	cactgcataa	ttctcttact	1920
gtcatgccat	ccgtaagatg	cttttctgtg	actggtgagt	actcaaccaa	gtcattctga	1980
gaataccgag	ccggcgacc	gagttgctct	tgccccgct	caatacggga	taatagtgt	2040
tgacatagca	gaactttaaa	agtgtctatc	attggaaaac	gttcttcggg	gcgaaaactc	2100
tcaaggatct	taccgctggt	gagatccagt	tcgatgtaac	ccactcgtgc	acccaactga	2160
tcttcagcat	cttttacttt	caccagcgtt	tctgggtgag	caaaaacagg	aaggcaaaat	2220
gccgcaaaaa	aggggaataag	ggcgacacgg	aaatggtgaa	tactcatact	cttccttttt	2280
caataattatt	gaagcattta	tcagggttat	tgctcatga	gcggatacat	atttgaatgt	2340
atthagaaaa	ataaacaat	aggggttccg	cgcacatttc	cccgaaaagt	gccacctgac	2400
gtctaagaaa	ccattattat	catgacatta	acctataaaa	ataggcgtat	cacgaggccc	2460
tttcgtctcg	cgcgtttcgg	tgatgacggt	gaaaacctct	gacacatgca	gctcccgag	2520
acgggtcacag	cttgtctgta	agcggatgcc	gggagcagac	aagcccgtca	gggcgcgtca	2580
gcgggtggtg	gcgggtgtcg	gggctggctt	aactatgctg	catcagagca	gattgtactg	2640
agagtgcacc	atatgcggtg	tgaaataccg	cacagatgcg	taaggagaaa	ataccgcctc	2700
aggcgaaatt	gtaaacgtta	atattttgtt	aaaattcgcg	ttaaatat	gttaaatacag	2760
ctcaattttt	aaccaatagg	ccgaaatcgg	caaaatccct	tataaatcaa	aagaatagac	2820
cgagataggg	ttgagtgttg	ttccagtttg	gaacaagagt	ccactattaa	agaacgtgga	2880
ctccaacgtc	aaagggcgaa	aaaccgtcta	tcagggcgat	ggcccactac	gtgaaccatc	2940
acccaaatca	agttttttgc	ggtcgagggt	ccgtaaagct	ctaaatcgga	accctaaagg	3000
gagccccga	tttagagctt	gacggggaaa	gccggcgaa	gtggcgagaa	aggaaggga	3060
gaaagcgaaa	ggagcgggag	ctagggcgct	ggcaagtgt	gcggtcacgc	tgcgcgtaac	3120
caccacaccc	gccgcgtta	atgcgccgt	acagggcgcg	tccattcgcc	attcaggctg	3180
cgcaactgtt	gggaagggtc	atcgggtcgc	gctcttcgc	tattacgcca	gctggcgaaa	3240
gggggatgtg	ctgcaaggcg	attaagtgtg	gtaacgccag	ggttttccca	gtcacgacgt	3300
tgtaaaacga	cggccagtga	attgtaatac	gactcactat	agggcggaatt	caaaaaaccc	3360
ctcaagaccc	gttttagagg	cccaaggggt	tatgctagt	aattctgcag	ggtaccggg	3420
gatcctctag	agatccctcg	acctcgagat	ccattgtgct	ggaaagcctt	tgcaaggctg	3480
gcaagccacg	tttgggtggt	gcgaccatcc	tccaaaatca	acaagtttgt	acaaaaaagc	3540
aggctatgct	aagtacatgt	cgattgcgta	cgcgttcgta	atgttggctg	tgttagtgcg	3600
taccagcgtt	caaattgttc	tcgagagtgc	gtttttacat	tatcccttca	tcctgattac	3660
gacaattttc	agctgttctc	gctcctacat	ctctcttcat	tgtcacaatg	gtcggaatct	3720
tcttctttgc	tgcatgtctt	catccaaaag	aattcacgaa	tattatccat	ggtgtcgtat	3780
tcttctctcat	gattccatct	acatatgtgt	tcctcacttt	atattcgctc	atcaatctca	3840
acgttatcac	gtggggaaact	cgtgaagctg	tcgctaaggc	aacgggacaa	aagacgaaaa	3900
aagcgcctat	ggaacaattt	atagacagag	tgattgatat	tgtgaaaaag	ggattcagat	3960
taatcagttg	tcgggagaag	aaggaacatg	aagagagacg	agagaaaatg	gaaaagaaaa	4020
tgagagaaat	ggagctagcc	ttgagaagta	ttgaggttat	ctttaacttt	agaaatgtga	4080
aattaataat	ttattttcag	agtgggtgccg	acgtgaagaa	aattctcgat	gcaacagagg	4140
agaaggagaa	acgtgaagaa	gaaactcaaa	ctgcagattt	tccgattgaa	gagaacgtag	4200
agaagactca	aaaagagatt	cagaaggcaa	accgttatgt	gtggatgaca	agtcatagct	4260
tgaaagtttg	tgaacgagga	aaactgaaaa	gtgcggaaaa	ggttttctgg	aacgagctca	4320
tcaatgcata	tctgaaaccg	atcaagacga	cgccagctga	aatgaaagcc	gtcgccgaag	4380
gattggcttc	tctacgaaat	cagattgctt	tcactattct	tctcgttaat	tctcttcttg	4440
ctcttgccat	ctttttgatt	cagaaacaca	aaaatgtgct	cagcatcaag	ttctcgccaa	4500
tcagtaagca	atattacctt	tatgggtcaat	tcaaaaaatt	tgtttttttt	ttctagaaaa	4560
cttccgatgg	acgaaaatga	atgagatgac	tggaacaatac	gaggaaaccg	atgaaccatt	4620
aaaaatagat	ccacttgga	tggaattgt	tgttttcctt	ctaattatct	tttttgttca	4680
aactctcgga	atgcttctcc	a				4701

<210> 7

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide primer C04H5.6F

<400> 7

tgctcagaga gtttctcaac gaacc

25

<210> 8

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer C04H5.6R

<400> 8

caatgttagt tgctaggacc acctg

25

<210> 9

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer K11D9.2bF

<400> 9

cagccgatct ccgtcttgtg

20

<210> 10

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer K11D9.2bR

<400> 10

ccgagggcaa gacaacgaag

20

<210> 11

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer Y57G11C.15F

<400> 11

accgtggtac tcttatggag ctcg

24

<210> 12

<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer Y57G11C.15R

<400> 12

tgcagtggat tgggtcttcg

20

<210> 13

<211> 52

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer T25G3.2F

<400> 13

ggggacaagt ttgtacaaaa aagcaggcta tgccaagtac atgtcgattg cg

52

<210> 14

<211> 52

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer T25G3.2R

<400> 14

ggggaccact ttgtacaaga aagctggggtt ggagaagcat tccgagagtt tg

52